

THE DEVELOPMENT OF THE HAMSTER METANEPHROS CORRELATED WITH GENERAL BODY GROWTH AND COMPARED WITH THE METANEPHROI OF OTHER SPECIES

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TWO FIGURES

INTRODUCTION

Although the general scheme of development of the mammalian kidney has long been known, the sequence of histological and cytological changes during embryonic growth for a single species has been studied only partially. Because of the size of the embryo and the extremely short gestation period, less than 16 days (Bruce and Hindle, '34; Bond, '45), the hamster offers an excellent form from which embryonic metanephric material may be obtained. In addition, it was deemed advisable to study the relationships between kidney differentiation, kidney growth and general body growth. In this respect, however, only a general comparison was made to determine if there are qualitative histological differences which may be correlated with quantitative growth variations.

The possibility of the significance of cyclic growth was early suggested by Minot (1891, '06), although he discounted small variations on the basis of chance. It has, however, continued to be a problem confronting students of embryonic growth (Brody, '25, '27; Robertson, '26; Murray, '25; Fowler, '37; Murphy and Dunn, '48). Schmalhausen ('26, '30) correlated possible cyclic changes in the growth of the chick

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with the differentiation of the organs. Organ-body weight studies have been carried on for many organs in various forms. Several organs of the cat have been statistically analyzed by Latimer ('48). Purdy and Hillemann ('50) have reported some indications of variation in the general growth rate for the hamster and Beatty and Hillemann ('50) found that the greatest number of ossification centers occurred at about the time of these variations.

The kidney is well suited for such a study because several reports have indicated at least a superficial relationship between body measurements and the size of the metanephros (Holl and Mac Gregor, '37; MacKay and MacKay, '27).

MATERIALS AND METHODS

Of the 402 hamster embryos from 54 litters utilized in this investigation, 190 were used for histological examination and 212 for the determination of weights and measurements. The females were bred between 8:00 and 11:00 p.m. and were killed at stages of 12 hours ($\pm .5$ hrs.) from the 9th to the 16th day post coitum.

Some of the embryos used for histological study were removed from the uterus and fixed in Zenker-formol. Other embryos were dissected in Locke's solution and only the metanephros was fixed. The specimens were then imbedded in paraffin, sectioned at either 8 or 12 μ and stained in Ehrlich's acid hematoxylin and erythrosin. Measurements of kidney elements are given in minima and maxima to indicate normal variation.

Embryos used for weights and measurements were dissected from the uterus in Locke's solution and immediately subjected to the following procedures:

- (a) the crown-rump measurements were taken by means of calipers and determined to the nearest 0.5 mm,
- (b) the volume measurements were obtained by placing the embryos in a vial of known volume and adding Locke's solution to a calibrated mark; the difference of fluid added, and the known volume at the mark

were taken as the volume of the embryo to the nearest 0.02 ml,

- (c) the wet weights were obtained by weighing the embryos in a vial on an analytical balance to the nearest tenth of a milligram ($\pm .2$ mg),
- (d) for the determination of the dry weights the embryos were then placed under an infra-red lamp at a distance of 16.5 cm (where the temperature ranged between 120°C. to 145°C.) until a constant weight was obtained; a constant weight was considered to be that which yielded a weight variation of not more than $\pm .2$ mg when readings were taken at half hour intervals; this yielded the dry weight and not the ash weight.

The embryos of at least two litters were used for each of the stages and included from 10 to 22 embryos per stage.

RESULTS

The first evidence of the metanephric kidney is noted at 9.5 days at which time the mesonephric duct is asymmetrical at its junction with the cloaca. On the dorso-lateral margin of the duct, the cells are considerably elongated and appear to be several layers thick. By 10 days the diverticulum has elongated and the distal end is surrounded by the mesenchymal blastema, extending from somite 27 to 29. The diverticulum remains closed proximally but the distal end has expanded to form the anlagen of the renal pelvis and the collecting elements. The diameters of the tubules and the length and the volumes of the kidneys for this and subsequent stages are found in table 2. The blastema increases in density by 10.5 days and has migrated cephalad to the region of somites 25 to 27. The expanded distal end of the diverticulum has given rise to anterior and posterior branches as primary collecting segments and, therefore, the ureter, renal pelvis and the collecting segments are now all distinctly represented. A slight aggregation of mesenchyme is present at the distal ends of the branched tubules and are the first

TABLE 1

Growth of embryo

STAGE	CROWN-RUMP MEAS.		VOLUME		WET-WEIGHT		DRY-WEIGHT		NO. OF CASES
	Avg.	Standard deviation	Avg.	Standard deviation	Avg.	Standard deviation	Avg.	Standard deviation	
<i>days</i>		<i>mm</i>		<i>ml</i>		<i>gm</i>		<i>gm</i>	
10	6.5	± 0.65	.032	± 0.10	.0341	± .0113	.0024	± .0007	13
10.5	7.9	± 0.50	.051	± .007	.0581	± .0083	.0045	± .0004	17
11	9.1	± 0.40	.107	± .015	.1007	± .0137	.0074	± .0012	16
11.5	10.1	± 0.56	.134	± .024	.1337	± .0207	.0105	± .0016	14
12	11.3	± 0.85	.165	± .026	.1833	± .0399	.0117	± .0043	18
12.5	13.2	± 0.64	.273	± .044	.3023	± .0477	.0253	± .0037	16
13	15.3	± 0.76	.554	± .056	.5593	± .0718	.0474	± .0073	14
13.5	16.3	± 0.97	.563	± .105	.5794	± .0926	.0525	± .0093	20
14	20.2	± 1.03	.947	± .138	.9839	± .1192	.0878	± .0130	16
14.5	22.0	± 1.07	1.221	± .178	1.2437	± .1411	.1213	± .0146	18
15	25.4	± 1.70	1.611	± .207	1.6093	± .2600	.1615	± .0165	22
15.5	27.1	± 1.66	1.902	± .296	1.9331	± .3006	.1902	± .0496	15
16	30.2	± 1.05	2.150	± .312	2.2370	± .3527	.2538	± .0362	10

TABLE 2

Growth of kidney

STAGE	DISTAL URETER DIA.		PROX. URETER DIA.		LENGTH URETER		ARCH. COLLECT. ELEMENTS		ST. COLLECT. ELEMENTS		RENAL PELVIS LENGTH		KIDNEY VOLUME
	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.	
<i>days</i>	<i>mm</i>		<i>mm</i>		<i>mm</i>		<i>mm</i>		<i>mm</i>		<i>mm</i>		<i>mm³</i>
9													
9.5													
10	.0315	.0380	.0315	.0380	.2726	.3260					.0630	.0714	.0037
10.5	.0252	.0315	.0189	.0252	.5178	.6541		.0252		.0441	.0756	.1099	.0127
11	.0315	.0441	.0189	.0315	.5996	.7359		.0252		.0441	.1090	.1635	.0274
11.5	.0315	.0441	.0189	.0315	.8125	1.0902		.0252		.0387	.1635	.2180	.0762
12	.0315	.0441	.0189	.0315	1.0356	1.2265	.0189	.0252	.0252	.0441	.2018	.2396	.1019
12.5	.0630	.0757	.0189	.0315	1.2810	1.3082	.0189	.0252	.0252	.0567	.2130	.2531	.2229
13	.0630	.0940	.0378	.0504	1.5864	1.7431	.0252	.0378	.0441	.0631	.2180	.2726	.4431
13.5	.0639	.0829	.0378	.0504	2.3715	2.4257	.0252	.0378	.0378	.0820	.3543	.4361	.8898
14	.1135	.1378	.0441	.0504	2.9981	3.2863	.0252	.0378	.0378	.0820	.4088	.4906	1.1196
14.5	.1450	.2018	.0964	.1109			.0315	.0378	.0378	.1135	.6814	.8177	2.6668
15	.2018	.2333	.1072	.1766			.0315	.0378	.0378	.1198	.6814	.8904	3.2619
15.5	.2089	.2439	.1072	.1829			.0378	.0441	.0441	.1324	.7359	.9539	5.9076
16	.2089	.2523	.1135	.1892			.0378	.0441	.0441	.1378	.9537	1.0902	9.1503

indications of the secretory elements derived from the blastemal mesenchyme.

The 11 day stage exhibits the initial formation of the renal capsule. Secondary branching of the collecting elements is the most extensive change aside from extensive growth.

Twelve day embryos show the separation of the metanephric tissue from the body wall and the formation of the primitive vascular system. The tubules are not conspicuously increased in number. Slight differences in diameter seem to be correlated with the differentiation of the straight and arched portions of the collecting segments as well as a difference in the epithelial cells which change from columnar in the former to cuboidal in the latter. The cellular aggregations in the blastema that represent the secretory elements have enlarged and, in some cases, have sent out growths from the ventral surface that curve laterally in the direction of the arched collecting segments. In some instances, these structures possess lumina and may be interpreted to represent the specific anlagen of the secretory portions.

Twelve and one half and 13 days involve growth stages with little advance in the individual structures except the formation of recognizable renal corpuscles and the recognition of cortical and medullary regions of the kidney. In addition, the first appearance of transitional epithelium in the ureter occurs at 13 days. In a few instances proximal segments with brush borders were found at 13.5 days.

At 14 days considerable undifferentiated mesenchyme remains at the cortical edge of the kidney and a gradient of developing secretory elements is evident in the cortex. The medulla is penetrated by many loops of Henle which extend almost to the renal pelvis. For the first time the ureter is open for its entire length and a few myoblasts can be observed in its walls.

The secretory elements at this stage show all phases of differentiation from the primordial type to those similar to the mature structures. Glomeruli and Bowman's capsules are fully developed in some instances. The secretory portions of

the tubules possess cuboidal or squamous-like neck portions and expand to enlarged convoluted portions with frequent evidence of brush borders. The loops of Henle are composed of cuboidal epithelium with typically flattened thin segments in some cases.

By the 14th day units of all the kidney elements can be found at a stage of differentiation comparable structurally to the functional units of the later non-embryonic stages. Stages from 14.5 days to birth are predominantly growth stages, with the size of the kidney approximately tripling in each of the 24 hour periods. There is also a considerable increase in the number of each of the individual elements during this interval. At birth the entire kidney is composed of a single cone-like renal pyramid which extends into the lumen of the renal pelvis. The cortex, composed of the pars radiata and pars convoluta, occupies about two-fifths of the kidney, while the medulla makes up another two fifths. Both cortical and medullary regions resemble the corresponding portions of the adult kidney.

The determination of the weights and measurements of the whole embryos is represented by table 1. The weights in grams and the lengths in millimeters are plotted against the embryo's age calculated from the time of observed copulation of the parents.

DISCUSSION

Metanephric development

A review of the results shows that the first indication of the appearance of the hamster metanephros involves the establishment of the ureteric bud at 9.5 days of post coital development. This is then followed by three phases of growth and differentiation (table 3). The first phase is characterized by the rapid growth and the differentiation into anlagen of the major general divisions of the kidney. The metanephric diverticulum grows dorso-laterally and penetrates the metanephric blastema which is established during the first 12 hours of development. During the next 12 hours,

the collecting elements may be distinguished as the future ureter, renal pelvis and collecting elements, while the secretory anlagen are present as aggregations of cells near the closed end of the collecting tubules. During this same period, the entire blastema migrates to a more anterior position.

The second phase of development is completed by 12 days post coitum. This level of differentiation is reached when the specific anlage of each of the units of the kidney is established.

TABLE 3
Critical periods of differentiation of the metanephros

KIDNEY ELEMENT	GROSS ANLAGE OF KIDNEY DERIVATIVE	SPECIFIC ANLAGE OF ELEMENT	DIFFERENTIATED STRUCTURE
	<i>days</i>	<i>days</i>	<i>days</i>
Total collecting portion	9.5 (M.D.*)	12	14
Ureteric epithelium	10 (M.D.*)	12	14
Ureteric substrate	10 (M.D.*)	12	14
Renal pelvis	10 (M.D.*)	12	13
Arched collecting segment	10.5 (M.D.*)	12	14
Straight collecting segment	10.5 (M.D.*)	12	14
Secretory portion	10 (B*)	12	14
Descending segment	10.5 (B*)	12	14
Ascending segment	10.5 (B*)	12	14
Proximal convolution	10.5 (B*)	12	13.5
Distal convolution	10.5 (B*)	12	14
Neck portion	10.5 (B*)	12	13
Bowmans capsule	10.5 (B*)	12	13
Glomerulus	10.5 (B*)	12	13

*M.D. — Derivative of metanephric diverticulum.

B — Derivative of blastema of metanephrogenous tissue.

The ureter begins to show the addition of two organized but undifferentiated strata external to the epithelial layer. The collecting elements can now be distinguished as arched and straight portions, while the anlagen of the secretory elements and glomeruli are represented by a proliferation of cells.

The third phase of development is initiated at the twelfth day and continues until the 14th day. During this period histological examination reveals cellular changes which indicate

that all the kidney elements now show a degree of differentiation which resembles that of the adult stage. From this stage to birth, differentiation continues but is characterized only by the duplication of the elements already present.

The overall process and sequence of differentiation in the hamster is the same as that found in the pig, cat, rabbit and human as determined by Huber ('05). Although early and late periods of differentiation were recognized by Huber, he combined the second and third phases. His interpretation, however, is based upon the more or less random selection of stages and the determination of histological structure from stages which did not necessarily represent the initiation of any one level of differentiation.

The appearance of the ureteric bud at 9.5 days and the presence of a well defined blastema by 10 days establishes the fact that the metanephros of the hamster appears earlier than in any other mammal previously investigated. McCrady ('38) reports that in the opossum the ureteric buds appear at 11.5 days, just 48 hours before parturition. In the rat, which most closely resembles the hamster in general development (Ortiz, '45), Torrey ('43, '45) reports the appearance of the blastema at 10 or $10\frac{1}{2}$ days.

The chick may be offered as an example of a form with an earlier appearing and more rapidly developing metanephros. Hurd ('28), Hanan ('27) and others have established the appearance of the metanephric anlage in the chick by the 8th day of incubation, at least $1\frac{1}{2}$ days prior to that of the hamster. When embryo age and the process of placentation in the mammal are considered, however, the chick metanephric anlage appears at a later relative time than in the hamster.

Some variation has been reported in the establishment of the metanephric anlage in the hamster. Ortiz ('45) reports that the blastema does not appear until the 10th day, but since it represents a more advanced stage of development it is comparable to an early 10 day stage as described in the present report. Boyer ('48) states that the metanephric

anlage appears at $9\frac{1}{4}$ days. This difference in development, however, is within the limits of variation which will be discussed later.

In the opossum (McCrary, '38) the developmental rate is considerably slower due to the process of discontinuous development. The metanephros of the $10\frac{1}{2}$ day hamster corresponds approximately to that of the $13\frac{1}{2}$ day opossum, while the 14 day hamster metanephros compares with that of the 7 day (post-parturition) opossum. In general, the development of the metanephros of the opossum requires, therefore, about twice the time that it does in the hamster. This interpretation is based on the initial appearance of tubules as well as on the differentiation of all kidney units. The extensive development of the mesonephros in the opossum (McCrary, '38) contrasted with its low level of development in the hamster, where only a few remnants of tubules remain at birth (Ortiz, '45) may in part account for the rapid development of the hamster metanephros.

In the rat, the rate of development of the metanephric kidney rapidly falls behind that of the hamster after the first appearance of the anlage. The early stages of development as described by Torrey ('45) and Swartz ('46), are similar to those of the hamster. The first 4 days of development in the rat corresponds to the first day in the hamster and, in later development, the 12th and 14th day stages in the hamster correspond to the 15th and 18th days in the rat.

Gersh ('37) reports a degree of differentiation in the pig between the 30th and 54th day of development which, in the present investigation, occurs between $13\frac{1}{2}$ and 14 days in the hamster. This is the period of cytological differentiation to a functional level in both forms. The metanephros of the 40 mm–65 mm cat and 14 mm–35 mm rabbit (Huber, '05) shows the same relationships as the metanephros of the 16 mm–20 mm hamster and also represents the same period of cellular differentiation.

The rapidly developing chick metanephros shows differentiation of all units by the 11th day (Hanan, '27; Hurd, '28 and

Gersh, '37). The chick kidney therefore requires only about two-thirds of the developmental period of the hamster from blastemal formation to the cytological changes which occur in the loops of Henle.

Comparison of the cellular nature of the developing and fully differentiated structures in the hamster kidney failed to show any variation in cellular morphology from those described by Huber ('05) or other investigators in various mammalian forms. The staining reaction of the distal convolutions appears to be basophilic rather than eosinophilic as described by Huber. This, however, is in accord with the observations of Cowdry ('44). In the hamster, the level of the development of the glomerulus seems to be at a more advanced stage when compared with the secretory tubules. In this respect, it compares more closely with the condition found in the kidney of the human and pig rather than with that of the cat or the rabbit.

Huber ('05) noted a difference in the size of the secretory tubules, for those which represent the initially differentiated units were larger than those subsequently formed. Measurements of the hamster tubules show no significant difference in this respect but support Huber in his contention that the first formed tubules do not atrophy during the later stages of development.

Functional differentiation in the hamster is apparently completed between $13\frac{1}{2}$ and 14 days, since at this time, all functional secretory units have differentiated and the metanephric duct possesses a lumen for its entire length. A comparison of the appearance of the secretory elements in other forms at the probable time of initiation of function is used as a criterion for predicting the first functional stages in the hamster kidney. Hanan ('27) and Hurd ('28) found that vital dyes in the chick, were eliminated and accumulated into the kidney tubules when their brush borders became differentiated (11-12 days). Sandstrom ('35) found similar results in the duck between 13 and 14 days of incubation.

Gersh ('34, '37) and Gersh and Stieglitz ('34) utilized ferrocyanide and phenol red to detect the earliest possible function in the metanephros of the rabbit, cat, chick and pig. The cellular appearance of the kidney tubules, at that time when these substances were first detected, is comparable to that of the hamster kidney at the 14th day of development.

A COMPARISON OF GROWTH AND DIFFERENTIATION

The variations of measurements and levels of differentiation may be due to the variation in implantation time of ova of the same litter. Although Graves ('45), Venable ('46) and Ward ('48) place hamster implantation at 4 days, Ward found variation, up to 8 hours, in the early blastocyst and morula stages. In addition, differences of several hours may exist between copulation and ovulation times. In the present investigation, copulation took place over a period of several hours during the evening preceding ovulation.

The results show a rapid change in the average growth of the hamster kidney during a period of 12 hours. When an 8 hour variable is considered in the light of these average changes, there is indication of considerable variation in the size and relative stage of development. This fluctuation is supported by the measurements of kidneys and entire embryos during a period of rapid growth or extensive differentiation (tables 1, 2, 3).

Daily increments in the volume of the metanephros (fig. 1) show that there is a period of growth depression from $11\frac{1}{2}$ to 12 days and from $13\frac{1}{2}$ to 14 days. It is during the first of these lags that the specific anlagen of the various parts of the metanephric kidney is laid down and during the second lag that the specific units differentiate to resemble adult structures.

The gross body measurements (table 1) show periods of growth depression in the form of reduced daily increments: one that ends at the 12th day, and one that is present at $13\frac{1}{2}$ days with a sudden rapid growth by the 14th day. The body

weights and measurements, therefore are also correlated fairly closely with the differentiation of the excretory system. Purdy and Hillemann ('50) found, in the hamster, that similar growth depressions occurred but at a slightly earlier

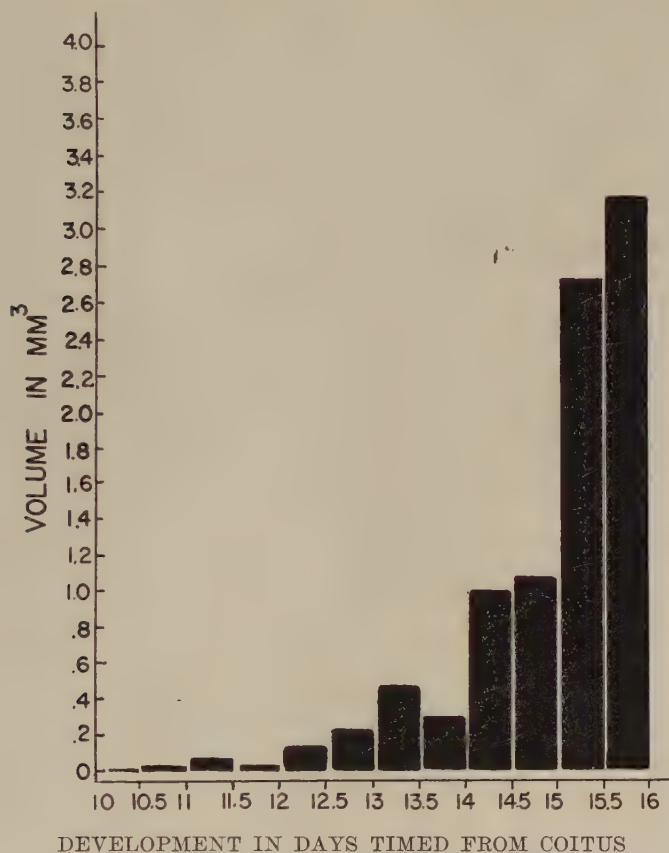


Fig. 1 Growth increments of metanephros by 12-hour intervals.

time. They, however, used 24 hour rather than 12 hour stages. Several other studies have indicated that the critical periods in development of specific organs are essentially in agreement with the present findings. Alessi and Swartz (personal communication) found that the developing liver of the hamster

first makes its appearance and shows distinct changes in accordance with the above times. The ultimobranchial body initially is found at the same time (Celestino and Swartz, '54), and the differentiation of centers of (Beatty and Hillemann, '50) osteogenesis are most active at these intervals.

In growth studies, the growth curves have been treated in various ways. Brody ('25, '27), Brody, Sparrow and Kibler ('26), Murray ('25), Robertson ('26) and Minot (1891) deal with the entire embryonic growth period as one, or a part of one, of the three cycles of growth in order to determine a mathematical master formula. Minot (1891), Murray ('25) and others considered the variation in the growth curve as accidental features and of no significance. Numerous other authors, however, have found small variations fairly consistent during the embryonic and post embryonic periods but have made no effort to correlate these variations with the histological picture at that time. Brody ('25, '27) seeks primarily to interpret the growth picture in its gross aspect and introduces the idea of "rhythmic growth." As one example of this he points out the growth rate changes in the chick from the 17th to 19th day of incubation and correlates these with accompanying changes in respiration. Murray ('25) stated that small variations were due to chance factors but later points out (Murray, '26a, '26b) changes in the growth curve at about 10 days and postulates that these are coincident with chemical changes which vary during growth.

In the post natal growth of the rat, Murphy and Dunn ('48) found 4 phases of growth which constituted a series of small cycles and subsequently were dependent upon the function, development or chemical processes present throughout the stages. Latimer ('48, '49), in studies dealing with the prenatal growth in the cat and dog, found that the percentage body weight of several organs had one or more periods of growth depression and growth acceleration. An examination of the specific study of the liver shows changes in percentage body weight at two points (Latimer, '39).

The most extensive correlation of body-organ measurements has been investigated in the chick, and some of these measurements have been correlated with differentiation of the tissue involved. Hasselbalch ('00), Byerly ('30), Schmal-

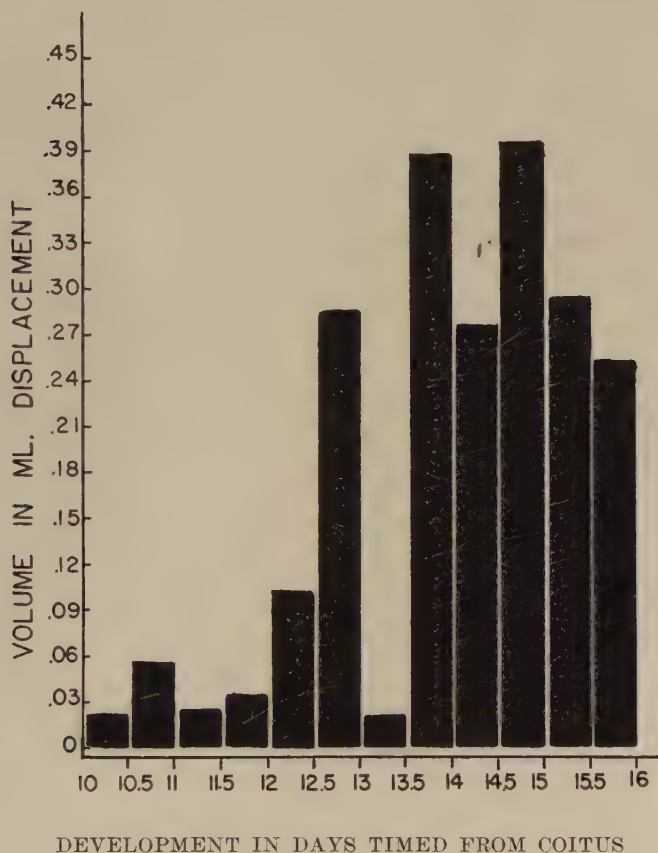


Fig. 2 Growth increments of embryo by 12-hour intervals.

hausen ('26) and others have determined the growth curve of the chick on the basis of daily growth increment, percentage growth, or instantaneous growth rate. An examination of their data shows a sharp decline in growth at about 11 to 12 days of development; again at 15 to 17 days; and

sometimes an earlier depression at about 8 days. Although complete agreement as to the exact time of growth depression is not evident, variation in breeds and methods of incubation must be considered.

In general, a review of the literature presents a preponderance of evidence that embryonic growth is not constant but varies at least in the rate of added mass.

Schmalhausen ('26) also carried out a determination of increase in relative size by a weight-volume method. He found that in the chick the percentage growth rates of the eye, lungs, heart, liver, mesonephros, metanephros and limb buds were synchronous with a growth depression period which coincided with that of the gross embryo. Although all organs were not affected to the same degree during each of the periods, sharp changes in the curve appear at the same stage of development. Schmalhausen and Stephanowa ('26) state that during the period of growth depression the organ forming process and the differentiation of these organs occur in the chick.

Schmalhausen ('26, '30) and Schmalhausen and Stephanowa (loc. cit.) also postulate that these periods of growth accelerations or retardations are due to substances present in the embryo which cause a self-inhibiting and self-accelerating effect. This is supported in general by the effect of variously aged tissue juices used in tissue culture. Carrel ('23) and Carrel and Ebeling ('23) made use of the sera of young and old chick tissues and found that the fluids of younger embryos produced a more rapid and extensive growth, but heating the fluid tended to destroy this substance. Fowler ('37) found that extracts obtained from chick embryos at 11, 14, and 17 days have the greatest growth promoting effect in tissue culture.

Finally, in examination of the daily increments in the embryonic growth of the rat, Stotsenbury ('15) shows depressions in growth at the 14th to 15th day and 18th to 19th day stages. Swartz ('46) reports that stages of metanephric

differentiation occur at 15 and 18 days in this animal. This again indicated a correlation between the growth of the entire embryo and the differentiation of the excretory system.

SUMMARY

1. One hundred-ninety hamsters from 9.5 to 16 days post coitum were examined histologically and crown-rump measurements, volume, wet weight, and dry weight determined for 202 embryos and 10 new-born animals.

2. The earliest appearance of the metanephros, among the known mammals, occurs in the hamster at 9.5 days post coitum.

3. Although the rate of metanephric development is much more rapid in the hamster, the general process is essentially the same as in other mammals.

4. All units for the kidney are established, at least structurally, between 13.5 and 15 days.

5. There is no indication of atrophy or a significant change in the size of the metanephric tubules during development.

6. The daily increments in growth of the kidney and gross body dimensions show synchronous depressions.

7. The differentiation of the metanephric elements approximately coincides with the lags in general overall body growth.

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THE RELATIVE GROWTH OF THE MAMMARY GLANDS IN NORMAL, OVARIECTOMIZED AND ADRENALECTOMIZED FEMALE MICE¹

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SEVEN FIGURES

The normal structure of the male mammary glands of several strains was described by Richardson ('51 and '53). Strain differences exist in the degree of development of the mammae. For example, the mammary glands of BALB/c males showed very little development in all of the mice examined. In contrast some of the glands in C57BR/cd males covered a large area, the ducts were wide and many small branches were present. There was a great variation in the size of the glands which ranged from rudimentary structures to those which covered an area of over 100 mm². In the large glands the duct pattern resembled that of virgin females except for the absence of the primary duct and nipple. The purpose of the present study is to determine if similar strain differences in the development of the mammary glands occur in gonadectomized female mice.

MATERIALS AND METHODS

Fifty-seven BALB/c females and 65 C57BR/cd females were gonadectomized three days after birth and were sacrificed at either 9 or 12 weeks of age. Twelve C57BR/cd females were gonadectomized when three weeks old and sacrificed at 12 weeks of age.

¹This investigation was supported in part by a research grant (PHS-C729) from the National Cancer Institute of the National Institutes of Health, Public Health Service.

Twenty-four C57BR/cd females, gonadectomized three days after birth, were adrenalectomized when 4 weeks old and a 15 mg pellet of desoxycorticosterone acetate was implanted subcutaneously. Four gonadectomized C57BR/cd females with intact adrenals were implanted with a pellet of desoxycorticosterone and sacrificed at 12 weeks of age.

The following normal females were used as controls: 30 BALB/c 9 weeks old, 23 BALB/c 12 weeks old, 10 C57BR/cd 4 weeks old, 30 C57BR/cd 9 weeks old, and 23 C57BR/cd 12 weeks old.

The diet of the mice consisted solely of Purina Laboratory Chow.

The uteri of all gonadectomized females were examined. Undeveloped uteri indicated the absence of ovarian hormones.

The procedure for preparing the skins and making whole mounts was described in a previous paper (Richardson, '51). Whole mounts were made of one of the third pair of glands in each series and then photographed. The outlines of the glands were traced on mm² graph paper and area was determined.

OBSERVATIONS

The mammary glands of all of the animals were examined under the dissecting microscope. A few general observations may be presented. (1) In gonadectomized BALB/c females 9 weeks old the area of the second pair of glands often exceeded that of the third pair but the ducts were very slender and few branches were present. In 12-week-old gonadectomized females the area of the glands was similar to that of gonadectomized females 9 weeks of age. (2) In C57BR/cd gonadectomized females the glands showed a marked increase in size between 9 and 12 weeks of age. (3) Twelve C57BR/cd females were gonadectomized at three weeks of age and sacrificed at 12 weeks of age. The mammary glands were similar to those of mice gonadectomized soon after birth. (4) The mammae of 30 normal BALB/c females 9 weeks old were examined. In many of these animals the third pair of glands was small; numerous end buds were present indicating the

beginning of a period of rapid growth. (5) The mammae of 30 normal C57BR/cd females 9 weeks old were studied. The third pair of glands developed at an earlier age than in most of the BALB/c females; no small glands were found.

The microphotographs show the morphology and extent of development of mammary glands of normal, gonadectomized, gonadectomized and adrenalectomized mice. In the gonadectomized BALB/c females the ducts of the glands are narrow and comparatively few branches are present. Figure 2 is a photograph of one of the largest glands of a mouse 12 weeks old. Many of the mammae of gonadectomized C57BR/cd mice have narrow ducts with few branches (fig. 3). The best developed glands in gonadectomized C57BR/cd females have wide ducts with many branches (fig. 6). Some of the large glands of the ovariectomized mice have narrower ducts than in the one pictured. A mammary gland of a normal C57BR/cd female is shown in figure 5. Numerous lateral buds are present in some of the normal females 12 weeks old; profuse budding does not occur in the gonadectomized mice. The largest glands of the gonadectomized and adrenalectomized mice (fig. 7) are similar to those of mice which have been gonadectomized only. A third mammary gland of a normal C57BR/cd female 4 weeks old is shown in figure 4. No glands are larger than this in the 10 mice sacrificed at this age.

Measurements were made of the third mammary glands of normal, ovariectomized, ovariectomized and adrenalectomized females. Figure 1 gives the distribution of the size of the glands and table 1 gives the mean, standard deviation and standard error for each of the 7 groups. All glands in BALB/c gonadectomized females were small at both 9 and 12 weeks of age. In contrast the glands of the normal BALB/c females were significantly larger ($t = 175$; $p < 0.001$) at 12 weeks of age. The glands of C57BR/cd gonadectomized females at 9 weeks of age show a wide variation in size. Considerable increase in size of the glands occurred between 9 and 12 weeks of age. A comparison of gonadectomized and normal C57BR/cd females 12 weeks old shows that the gonad-

ectomized females had significantly smaller glands ($t=7.9$; $p < 0.01$). While this difference is very significant it is not as great as the size difference between gonadectomized and normal BALB/c females. A comparison of the glands of

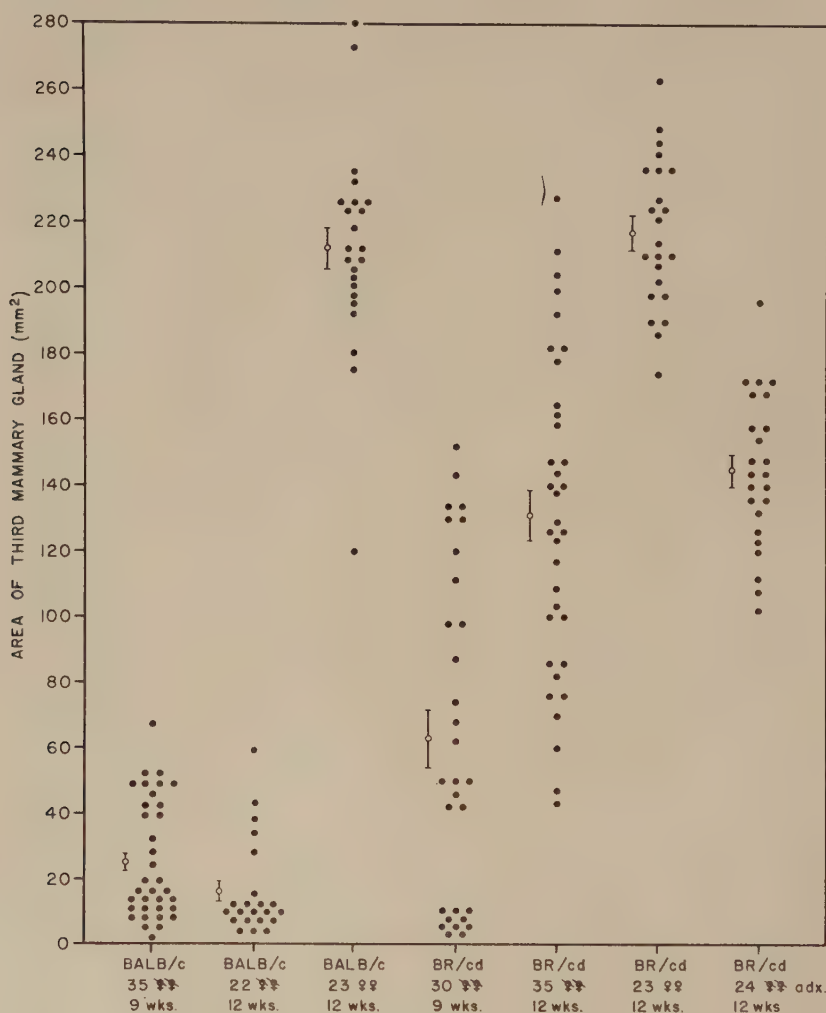


Fig. 1 Areas of third mammary glands of normal and gonadectomized BALB/c females and of normal, gonadectomized, gonadectomized and adrenalectomized C57BR/cd females. The mean (small circle) and standard error (vertical line) is indicated for each group. Cancelled ♀♀ = gonadectomized females; cancelled ♀♀ adx. = gonadectomized and adrenalectomized females.

C57BR/cd gonadectomized females and C57BR/cd gonadectomized and adrenalectomized females shows there is not a significant size difference ($t=1.3$; $p=0.2$). A pellet of desoxycorticosterone was implanted into 4 C57BR/cd gonadectomized females with intact adrenals. There was no indication that the pellet stimulated growth of the glands.

TABLE 1

MICE	TREATMENT	NO.	AGE IN WKS.	AREA OF THIRD MAMMARY GLAND (MM ²)		
				Mean	Standard deviation	Standard error
BALB/c	Gonadectomized	35	9	25	± 17.7	± 3.0
BALB/c	Gonadectomized	22	12	16	± 15.2	± 3.2
BALB/c	Normal	23	12	212	± 31.1	± 6.4
C57BR/cd	Gonadectomized	30	9	63	± 50.7	± 9.2
C57BR/cd	Gonadectomized	35	12	131	± 47.8	± 8.1
C57BR/cd	Normal	23	12	217	± 22.1	± 4.6
C57BR/cd	Gonadectomized and adrenalectomized	24	12	145	± 22.6	± 4.6

DISCUSSION

In previous studies of the mammary development of normal male mice (Richardson, '51 and '53) it was found that there was very little development of mammae in BALB/c males, whereas in C57BR/cd males some glands covered a large area, and the ducts were wide and well branched, indicating both a significant strain difference in mammary development and that development may take place without the influence of ovarian hormones.

The present investigation shows a correlation between the development of the mammary glands in male mice and that of gonadectomized female mice. At 12 weeks of age the mammary glands of BALB/c gonadectomized females were very much smaller than those of normal females; the ducts were very slender and few branches were present. A few of the C57BR/cd gonadectomized females at 12 weeks of age covered an area equal to that of normal females. In some of the large

glands the ducts were as wide as in the normal animals, but often the ducts in the region of the nipple were wide while those extending toward the lateral regions were slender with few branches. Another similarity between the mammary glands of C57BR/cd males and those of gonadectomized females was the great difference in the size of the glands, ranging all the way from small glands to large glands. In general, where one large gland was found in the gonadectomized female the other glands tended to be well developed but this was not always true.

Other investigators have described the mammary glands of gonadectomized females. These descriptions seem to correspond more nearly to what was found in BALB/c rather than those in C57BR/cd females.

Woolley, Fekete and Little ('40) stated that some of the mammary glands of C57BL females gonadectomized at birth remained rudimentary and were comparable to those of normal male mice while others, sometimes in the same mouse, developed systems with long but slender ducts. Fekete, Woolley and Little ('41) said that in general glands of gonadectomized DBA females remained rudimentary for about 8 months and were comparable in size to the gland system of a three weeks old female. After that period the ducts gradually lengthened and the formation of end bulbs occurred. The uterus remained undeveloped until 6 to 7 months of age. In all of the animals killed after 3 to 4 months of age the suprarenals showed characteristic changes which lead to the formation of nodular hyperplasia.

Yokoyama and Syoda ('53) used the Kasukabe strain of mice. Female mice were gonadectomized at 20 days of age and sacrificed at 35 and 40 days of age. In the normal females very rapid development of the duct system occurred between 25 and 35 days of age and the index of the mammary duct area of mice 30 days of age was approximately 8 times as large as those of 25 days. Development of the mammary ducts of the females ceased after gonadectomy and resembled the glands of normal females 20 days of age. They concluded

that probably sex hormones were secreted from the ovary even in immature stages and that the rapid development of the gland is mainly due to ovarian hormones. Our findings are not in agreement with the statement that development of the mammary ducts of the females ceased after castration.

The present observations on gonadectomized females not only supports the previous conclusions derived from studies on male mice, that large mammary glands may develop without stimulation by ovarian hormones but it further shows that adrenal stimulation is not necessary to produce large glands.

In the C57BR/cd mice which were gonadectomized and adrenalectomized the mammary glands were similar to those of mice that were gonadectomized only except that there were no small glands found in the adrenalectomized mice. While it is possible that the pellet of desoxycorticosterone had a slight stimulating effect on the ducts there was no evidence of any stimulation in females gonadectomized only which were implanted with a pellet of desoxycorticosterone; a small number of mice was used in this series.

Previous experiments on the effect of adrenalectomy on mammary development have given rather conflicting results. Reeder and Leonard ('44) found that adrenalectomy in normal or castrated immature male rats resulted in an increased number of lateral buds on the duct system and in some cases increased end bud growth. Cowie and Folley ('47) observed that regressive changes frequently but not invariably occurred following adrenalectomy of hooded Norway rats of both sexes which were gonadectomized at weaning age. They concluded that there was little evidence that the adrenal cortex plays an outstanding or essential role in the normal development of the mammary gland.

Van Heuverswyn, Folley and Gardner ('39) stated that desoxycorticosterone promoted mammary duct growth in immature male mice. On the other hand Chamorro ('45) observed no more than slight or irregular mammary duct stimulation in mice in response to desoxycorticosterone.

Thus it has been demonstrated that the mammae of mice may develop into large glands without stimulation by either hormones from the ovaries or adrenals. Possibly the pituitary hormones stimulate the growth of the mammary ducts in the ovariectomized females but no adequate explanation can be given for the great variation in the size of the glands.

SUMMARY

Mammary glands of 122 female mice of BALB/c and C57BR/cd strains, gonadectomized at three days of age, sacrificed and observed at 9 and 12 weeks of age were compared with glands of normal females of the same age. Twenty-four C57BR/cd females were also gonadectomized after birth, adrenalectomized at 4 weeks of age and their mammary glands were examined after sacrifice at 12 weeks of age. There was no development of the uterus in any of the gonadectomized animals.

The glands of BALB/c gonadectomized females remained small. At both 9 and 12 weeks of age the ducts were very slender and had few branches. Those of C57BR/cd similarly treated showed a wide range of development including some which resembled those of normal females of the same age having wide ducts with many branches. A great increase in size occurred after 9 weeks. Gonadectomy followed by adrenalectomy had no greater effect than gonadectomy alone in C57BR/cd females.

Measurements of the third mammary glands of normal females showed a significant strain difference. In the BALB/c mice the greatest development occurred after 9 weeks whereas in C57BR/cd females no small glands were observed in 9-week-old animals. The third pair of glands of both strains were similar in size at 12 weeks.

ACKNOWLEDGMENT

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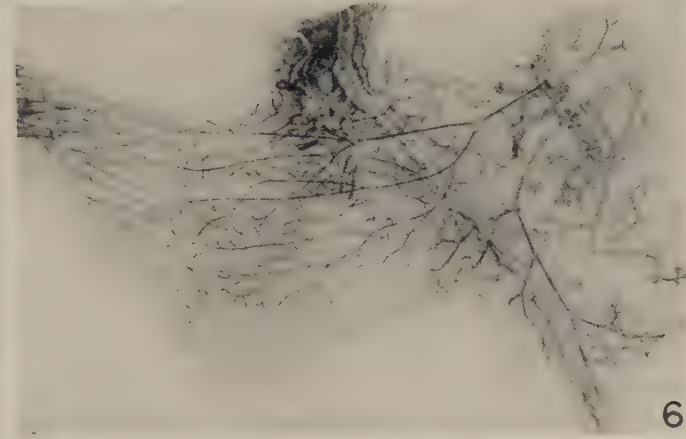
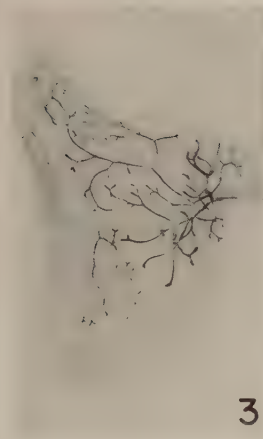
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PLATE 1

EXPLANATION OF FIGURES

All figures are photomicrographs of whole mount preparation of mammary glands of female mice $\times 3.5$.

- 2 Third mammary gland of BALB/c gonadectomized female 12 weeks old. Area is 43 mm².
- 3 Small third mammary gland of C57BR/cd gonadectomized female 12 weeks old. Area is 43 mm².
- 4 Third mammary gland of C57BR/cd normal female 4 weeks old. Area is 21 mm².
- 5 Third mammary gland of C57BR/cd normal female 12 weeks old. Area is 244 mm².
- 6 Large third mammary gland of C57BR/cd gonadectomized female 12 weeks old. Area is 227 mm².
- 7 Large third mammary gland of a gonadectomized and adrenalectomized C57BR/cd female 12 weeks old. Area is 195 mm².



A CASE OF LEFT-SIDED VERMIFORM APPENDIX, CECUM AND ANOMALOUS COLON

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SEVEN FIGURES

INTRODUCTION

The vermiform appendix is a finger-like structure first described as a visceral organ in 1524 by Berenger (Singer, '26). It projects from a pouch-like structure, the cecum, and according to Goss ('54) varies in length from 2 to 20 cm, the average being 8.3 cm. The base diameter is 6 mm and its apex measures 5 mm. It is situated normally in the right iliac fossa. The latter, however, is not always true. As a result of abnormal development, according to Kelly ('09), there may be marked variability in the position of the appendix, the cecum, the colon and other visceral structures in the thorax and abdomen.

In the comparatively few cases of left-sided appendix on record and which have come to light principally during an operation for appendicitis, literature offers several contributing factors: left-sided appendix and cecum have been shown to be due to transposition of the viscera (Willis, '25), failure of peritoneal fusion or attachments (Harvey, '18), incomplete intestinal rotation (Newburger, '33), congenital non-descent of colon (McKnight, '44), according to Deaver ('05), Lennander in 1895 reported a case of a boy, aged 16, who had "cecum and appendix situated in the left hypochondriac region lying against the spleen." Lichstein ('42) reported a case of "probable" left-sided appendix in a white female, aged 67, due to partial non-rotation of the colon. He

reviewed the "surgical literature for the past twenty years, both in the periodicals and surgical systems as to the frequency of left-sided upper quadrant appendicitis, and was unable to find a report of a similar case."

An examination of the literature on this subject further reveals that along with left-sided appendix situs inversus is infrequently co-incidental. Prescott ('44) reports situs inversus totalis or abdominus. According to Chundnoff and Shapiro ('39), situs inversus occurred in from one in 500 to one in 5,000 persons. Haddon ('45) reported a case of appendicitis of a soldier, aged 19, whose appendix occupied the "left lower abdomen with its tip reaching the recto-vesical pouch." The cecum also was situated in the left iliac fossa. Yet, the viscera in the thorax and abdomen at autopsy showed no signs of situs inversus.

In view of the fact that such anomalies are misleading anatomically, clinically and surgically which tends to increase the operative risk, coupled with the scarcity of reported cases, the object of this paper is to place on record an occurrence of left-sided appendix and related anomalies. As in the case reported by Haddon, there was no evidence of situs inversus.

DESCRIPTION OF THE CASE

During the routine dissection of a body in the Anderson Gross Anatomy Laboratory, Meharry Medical College, an anomaly of the appendix, cecum and colon was discovered. The subject, an adult Negro male, had died in a state institution for the aged and had been embalmed immediately after death. There were no signs of scars as a result of surgical operation on the abdomen before or after death, nor were there observed any external scars or deformities in any other regions of the body.

The abdomen was entered by means of 4 incisions. The first extended from the ensiform process of the sternum through the linea alba to the symphysis pubis; the second, from the anterior superior spine of the ilium, traversing Poupart's ligament, to meet the median incision at the sym-

physis pubis; the third, transversely from the tip of the ensiform process to the posterior axillary line; and the 4th, transversely from the umbilicus to the lateral aspect of the abdomen. Thus, 4 flaps were produced, two on the left and two on the right, which when reflected exposed the abdominal viscera in situ (fig. 5).

THE VERMIFORM APPENDIX

A careful observation was made of the ascending colon, situated in the right iliac fossa and the appendix was found to be absent. Upon further exploration of the abdomen, the vermiform appendix and cecum were found in the left iliac fossa (figs. 5, 6). The appendix was situated on the medial surface of the cecum between the latter and the small intestine at the ileocecal junction. It measured 6 cm from its base to the apex and 2 mm above where the terminal portion of the ileum enters the cecum. It was held to the cecum by a triangular shaped strip of peritoneum, the appendicular peritoneum, which blended with the peritoneum of the ileum where it enters the cecum. There was a right superior ileocecal fold and a left inferior ileocecal fold. Situated between these two folds was the ileocecal fossa which was 3 cm wide and 2.5 cm in depth. Located on the right and left side of the appendix there were two small pouches or recesses. These pouches were 2 cm wide and 1.5 cm in depth on each side, and were formed by the peritoneum at the interval of the terminal part of the ileum, left surface of the cecum, and upper surface of the anomalous intestine where it leaves the cecum on the left side (fig. 6).

THE CECUM

The cecum may be described as a blind pouch of the ascending colon, situated below the entrance of the ileum. Its average length in adult human subjects is between 6 and 7 cm and its breadth is 8 cm. However, in this case, the cecum, situated in the left iliac fossa, measured 11 cm in length

and 6 cm in breadth. It was lying on the iliac fascia, the terminal portion of the descending colon and the beginning or upper part of the sigmoid colon to which latter two structures it was attached by the peritoneal ligament and the lower part of the iliopsoas muscle (fig. 6).

At the beginning of the ascending colon in the right iliac fossa, a sacculaton or diverticulum was observed which was 6 cm in length and 5 cm in breadth. It was attached to the dorsal body wall by peritoneum. There were no visible signs of a vermiform appendix, but it resembled a cecum and conformed in a large measure in that it had a blunt conical form without appendix as reported by Huntington ('03), (figs. 6, 7). Records reported a few cases of ceca without the appendix. Gladstone ('15) reported a case of a female with an "asymmetrical form of cecum without appendix." Green and Ross ('33) observed in an adult male a cecum without appendix. During the following year, Feldman ('34) reported a right-sided cecum without appendix in a girl aged 12.

EXTRA SECTION OF INTESTINE

Interposed between the cecum on the left and a rudimentary cecum or sacculaton on the right, there was an extra section

ABBREVIATIONS

1, 2 reflected flaps on the right side.	3, 4 reflected flaps on the left side.
R.Ab. M., recti abdominal muscles.	R.L.L., right lobe of the liver.
L.L.L., left lobe of the liver.	s., stomach.
T.C., transverse colon.	G.O., greater omentum.
S. L., small intestine.	Hemostat pointing to vermiform appendix.
P.P., parietal peritoneum.	V.P., visceral peritoneum.
C., cecum.	R.L., round ligament of liver.
F.L., falciform ligament.	D., diverticulum or sacculaton.
E.S.I., extra section of intestine.	A.C., ascending colon.
R.C.F., right colic flexure.	L.C.F., left colic flexure.
V.A., vermiform appendix.	R., rectum.
D.C., descending colon.	S. C., sigmoid colon.
S.F., sigmoid flexure.	P.d.m., primitive dorsal mesentery.
P.V.M., primitive alimentary canal.	P.m., dorsal mesentery.
V.m., ventral mesentery.	P.l., peritoneal ligament.
I.e.j., ileocecal junction of left.	

of large intestine. To the left of the cecum, about 5 mm below the ileum, it ran paralleled to the beginning part of the sigmoid colon to which it was attached by peritoneal strands. It then ran transversely across the lesser pelvis to join the ascending colon in the right iliac fossa. It was 12 cm in length and its diameter 5 cm and was situated at the interval

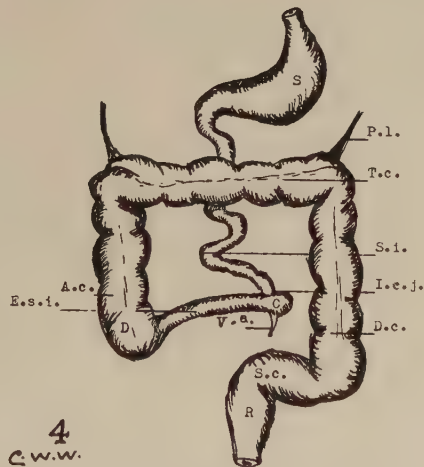
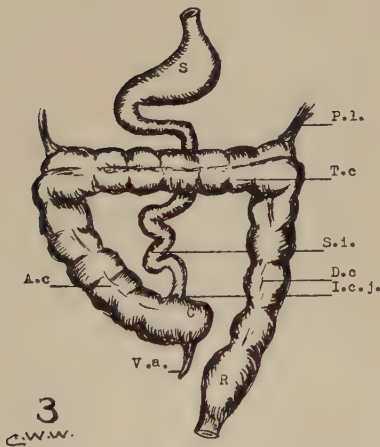
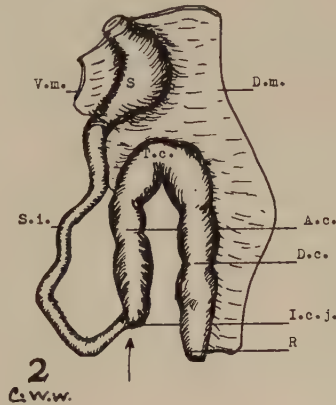
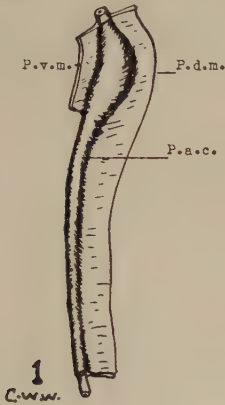


Fig. 1 Growth and extension of mesenteries and alimentary canal.

Fig. 2 Growth, extension and rotation of the alimentary canal and mesenteries.

Fig. 3 Growth, extension, differentiation and rotation and alimentary canal.

Fig. 4 Anomalous arrangement of the definitive alimentary canal.

where it joins the beginning part of the ascending colon in the right iliac fossa (figs. 6, 7). It was enclosed by peritoneum and attached to the posterior abdominal body wall and ran below in the pelvis similarly as does the transverse colon above.

Histological sections of the vermiform appendix, cecum, terminal portion of the ileum and of the extra section of intestine were made and stained with H and E. This technique revealed that the definitive vermiform appendix, the cecum and the ileum situated on the left, in this case, presented the normal histological picture. The extra section of intestine appeared to have had a structure similar to that of the large intestine. However, the muscular coats of the former appeared very thick.

DISCUSSION

The intestinal irregularities illustrated and found in this specimen are understandable on the basis of embryological development. Failure or improper attachment of the intestine by mesentery during intestinal rotation and elongation will produce abnormal development (figs. 1, 2). The evidence in this case suggests that the cecal end of the colon was fixed by the mesentery to the dorsal body wall on the left side (fig. 3). As the intestine elongated and rotated, the cecal portion failed to move to the right just below and across the duodenum, but remained stationary (fig. 4). However, connected to this arrested ileocecal part of the intestine on the left were the cecum, appendix, ileum and extra section of the large intestine which joined the ascending colon in the right iliac fossa.

SUMMARY

1. A review of literature reveals that the incidence of left-sided appendix occurs infrequently and that the occurrence of left-sided appendix and cecum without situs inversus is rare and is occasionally discovered during an operation for appendicitis.

2. A sacculaton or diverticulum was interposed between the beginning of the ascending colon and anomalous section of large intestine in the right iliac fossa.

3. The cause of left-sided appendix, cecum and anomalous large intestine was believed to be due to fixation of the mesocolon at the ileocecal junction.

4. Fixation of the alimentary canal at the ileocecal junction on the left side did not prevent normal rotation of the small intestine, ascending, transverse, descending and sigmoid colons.

I wish to acknowledge the assistance of Drs. R. H. Miller, former Professor of Gross Anatomy, University of Tennessee Medical College, now visiting Professor of Gross Anatomy, Meharry Medical College, and Gadson J. Tarleton, Jr., Chairman of the Department of Radiology, Meharry Medical College, during this study. In the course of this investigation, much encouragement has come from Dr. W. M. Bright, Messrs. M. H. Coleman and C. L. Calhoun, of the Department of Microscopic Anatomy. I am also grateful for the assistance of Mrs. M. Magalena Lauray, secretary and technician.

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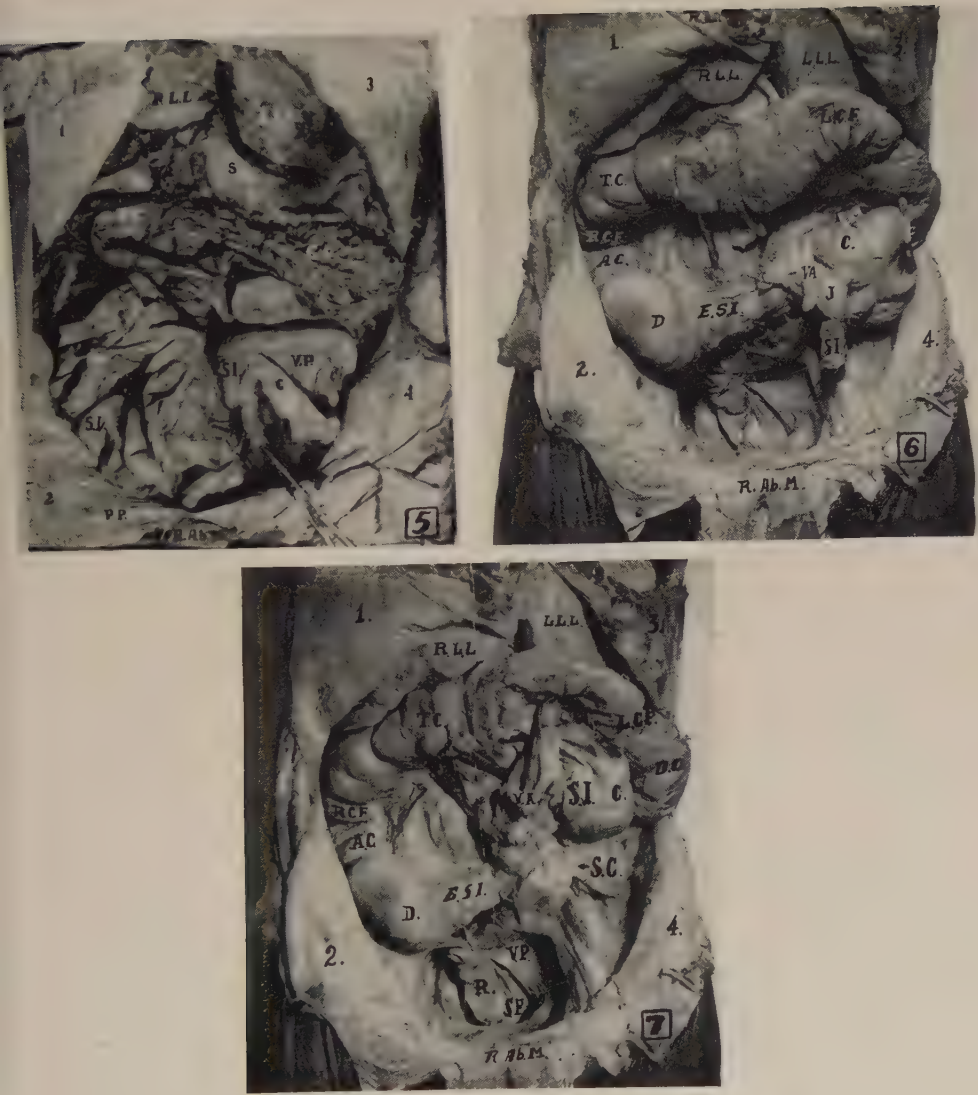
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PLATE 1

EXPLANATION OF FIGURES

- 5 Viscera in situ.
- 6 Abdominal cavity with small intestine and greater omentum removed.
- 7 Abdominal cavity with greater omentum, small intestine removed, Ileum, and Vermiform appendix displaced upward to show continued outline of large intestine.



SOME OBSERVATIONS ON THE GROSS ANATOMY
OF THE GENITAL SYSTEM AND TWO
ENDOCRINE ORGANS AND BODY
WEIGHTS IN THE
CHINCHILLA ¹

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FOUR FIGURES

The chinchilla is of unusual interest to the zoologist because it is one of the few survivors of a once successful group of small mammals. At one time the chinchilla and related forms were well distributed throughout the South American continent. Fossil remains of larger species of the Chinchillidae have been identified from the entire pampas area, but only two species, *C. brevicaudata* Waterhouse and *C. lanigera* Bennett, represent the genus *Chinchilla*; two genera of viscachas (*Lagostomus* Brookes and *Lagidium* Meyen) complete the family. Chinchillas are found in the wild state at present only in the timberline region of the Peruvian Andes and in certain isolated coastal areas. Fossil remains of this family have been discovered from rocks as early as the Oligocene. The three modern genera date from the Pleistocene, at the

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earliest, with *Lagostomus* the oldest and *Chinchilla* the youngest member of the triumvirate.

Less than a dozen *C. lanigera* individuals were brought into California by Mr. M. F. Chapman in 1923, these animals being the sole progenitors of the estimated half million or more chinchillas now in this country. A few additional *C. lanigera* have been imported from South America from time to time, as well as small numbers of the larger *C. brevicaudata*. The two species will hybridize; the hybrid males are reported to be completely sterile, while the females reproduce when backcrossed to either parental type. Those well acquainted with chinchilla breeding on the North American continent state that by far the major portion of animals throughout the country are representatives of the *lanigera* species.

Chinchillas in the University of Wisconsin colony⁴ have been maintained under uniform conditions and those included here were kept as part of the general herd for a period of not less than one month prior to sacrifice. Alfalfa hay, a commercial chinchilla pellet, and water are provided *ad libitum*; animal house temperature is stabilized at 65°F. $\pm$ 5°F. throughout the year.

All animals used in this study were two years of age or older with the exception of those used in constructing the growth curve. It is assumed that this sample is representative of the general *C. lanigera* population in this country; neither gross external abnormalities nor pregnancies were observed, and none of the individuals were reported by their donors to be sterile. Live body weights were measured to 0.1 gm on an Ohaus triple-beam balance equipped with an animal cage instead of a weighing pan. Chinchillas were killed by disarticulation of the skull from the spinal column. Organs were removed immediately, cleaned on moist bibulous paper and weighed on a Roller-Smith torsion balance to the nearest 0.1 mg. Weights were analyzed by standard statistical methods.

⁴ Animals for this colony were donated to the University by members of the National Chinchilla Breeders of America, Inc.

DISCUSSION

The genital system

Male

The reproductive system of the male chinchilla differs in several respects from the generalized mammalian type. Except at times of sexual excitation, the penis is directed posteriorly; it assumes a cranially oriented position at erection. Only the slightest evidence of a scrotal region is visible externally. Sparsely furred areas are occasionally found in some individuals on either side of the penis, although there is no evidence of a pendulous scrotum. Dissection reveals the presence of thin-walled scrotal diverticulae extending caudally along either side of the base of the penis. The gubernacula are attached broadly to the posterior walls of the scrotal sacs.

Internally, one is impressed by the large size of all elements of the genital tract, especially the seminal vesicles. The testes are located within the pelvic girdle, in their respective inguinal apertures, the cauda epididymides occupying the scrotum proper. A large fat body expands cranially from the anterior pole of each testis. The vas deferens emerges from the testicular hilum as a very fine tube which immediately coils to form the compact caput epididymis, embedded in the mesorchium; the caput extends the length of the testis.

The ductus deferens is helicoid as it leaves the caput epididymis but quickly becomes more intricately coiled to form the cauda epididymis; it finally emerges from the epididymal mass at the anterior end and loops cranially over the ureter and seminal vesicle. Continuing dorsally and posteriorly over the bladder and urethra, it fuses with the latter to form the urogenital duct in the region of the symphysis pubis. The cauda epididymis presents a bilobed appearance achieved as follows: the highly convoluted ductus folds sharply back upon itself, and since it gradually increases in diameter (rather abruptly at the posteriormost end of the

cauda), the visible portion of each of the two opposed lobes, as seen from the ventral aspect, appears to be composed of the vas deferens in either its largest or smallest diameters. The heavy gubernaculum is attached at the posterior flexure of the cauda epididymis.

The large seminal vesicles are attached to their parent ducts just distal to fusion of the latter with the urethra. Two

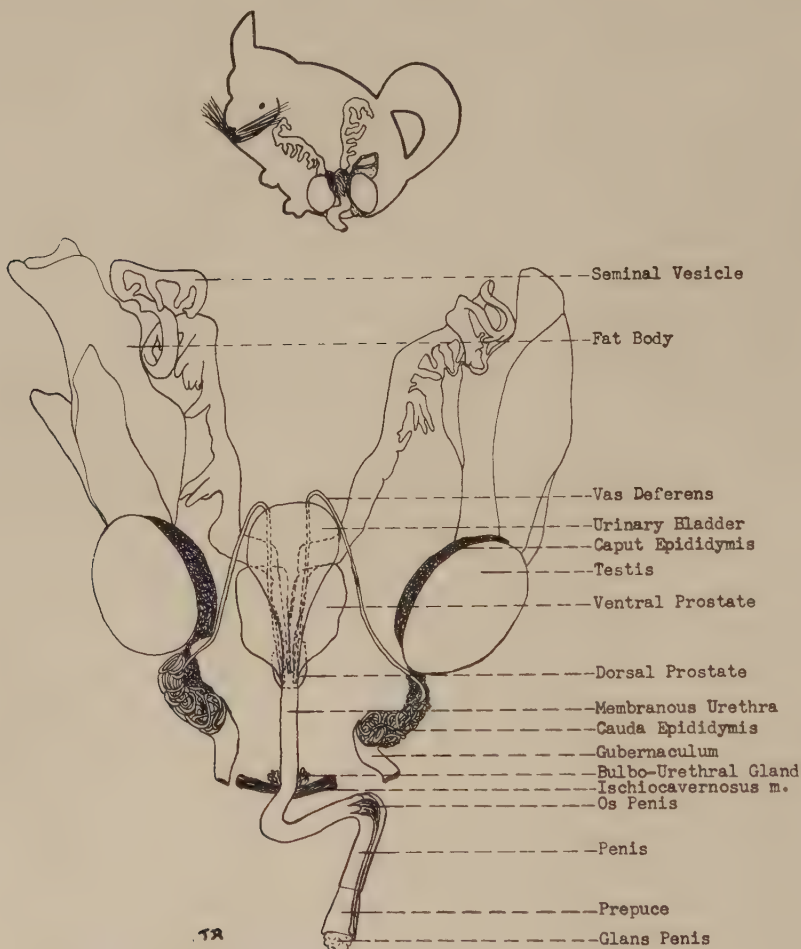


Fig. 1 Chinchilla Urogenital System. Male. Life size. Ventral aspect. The upper diagram represents the position of the various organs in the live animal when in the sitting posture.

large, highly vascular lobes of the ventral prostate gland accompany and surround the base of the seminal vesicles from near the region of their confluence to a point below the merger of the ductuli deferentia with the urethra. A small dorsal prostate is located in the region of this juncture. The fleshy membranous urethra continues caudally from the formation of the common urogenital duct to the posteriormost portion of the body cavity. Buried in the ischiocavernosus muscle at the base of the penis is the bulbourethral (Cowper's) gland.

The base of the penis is enclosed in a dense connective tissue sheath and the entire organ is thrown into an S-shape with the membranous urethra forming the outer loop. An os penis is located in the distal arm, originating in the tissue surrounding the corpora cavernosa of the transverse bar. Two alate flaps, one from either cavernous body, diminish in size and fuse distally to form a single, mid-dorsal prong extending through the prepuce and ending in a small bulb near the base of the glans. On erection the entire organ is extensible, the transverse portion being extruded outside the body to add to the length of the external penis; this extrusion also serves to reverse the resting position of the penis. Enclosed within the prepuce is a membranous glans penis which, during tumescence, becomes everted, forming a spongy, club-shaped head.

Weights were taken of testis pairs from 38 animals; the average weight per testis was found to be 2599.8 mg. No difference could be demonstrated between the weights of right and left gonads from the same animal; no significant differences were shown by weights of testes from animals of different ages (from two to eleven years).

Female

The genital tract of the female chinchilla is superficially similar to that of the more typical rodent. Each ovary is located lateral to the mid-point of the corresponding kidney, partially enclosed within a bursa which communicates with

the celom through a medial ostium (fig. 3 a). The expanded oviduct runs cranially for a short distance before turning caudally and becoming a fine, convoluted tube lying along the lateral border of the ovary. This tube broadens abruptly into a long uterus. The two uteri continue caudally toward the midline to an apparent junction in the region of the formation of the posterior vena cava. The uterine lumina do not

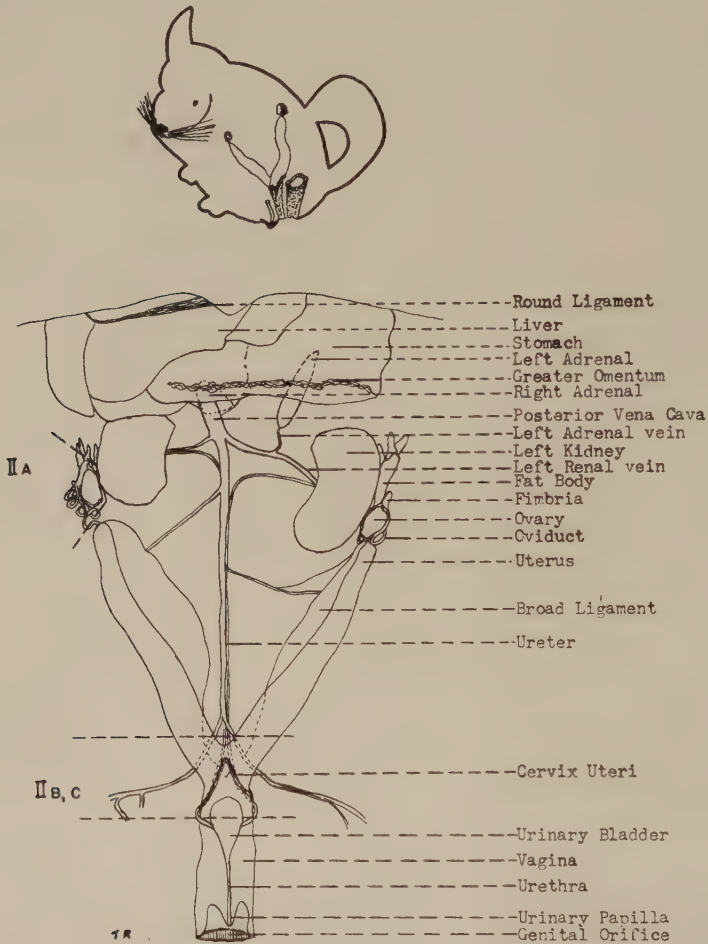


Fig. 2 Chinchilla Urogenital System. Female. Life size, Ventral aspect. The upper diagram represents the position of the various organs in the live animal when in the sitting posture.

fuse (uteri duplex), but enter the vagina by way of separate ora cervices (fig. 3 c). Urethra, vagina, and rectum pass through the pelvic girdle, retaining their separate identities. The urethra terminates in a urinary papilla ventral to the vagina. The vaginal aperture appears to be sealed in normal animals throughout diestrus, opening only at estrus and parturition. There is no evidence of a vestibule.

Twenty-two pairs of ovaries were weighed, the average weight being 25.7 mg per ovary. No difference was found be-

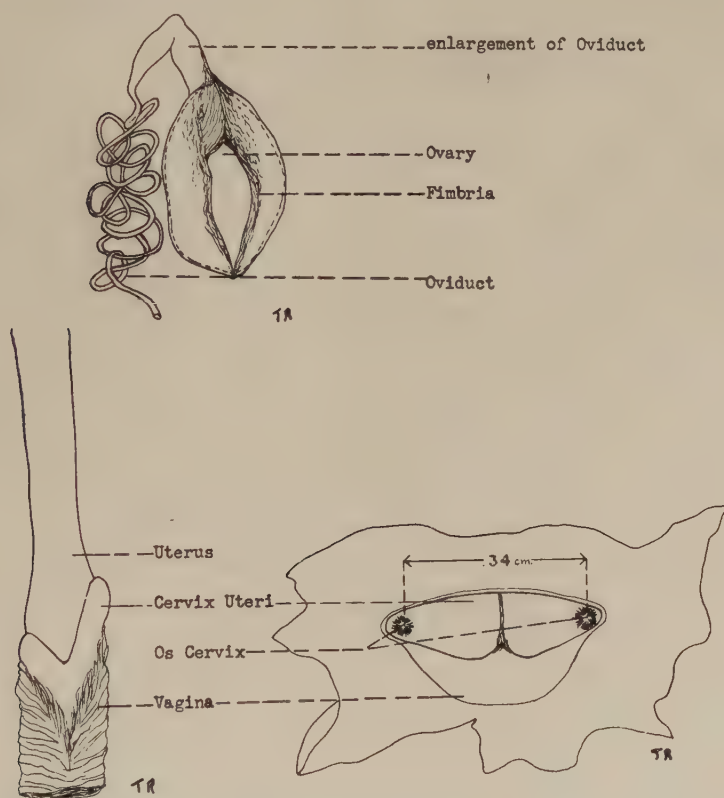


Fig. 3 Enlarged drawings of portions of the reproductive tract of the female chinchilla:

- a. Ovary, Oviduct and Fimbria. $\times 4$. Medial aspect.
- b. Cervix Uteri. $\times 4$. Left lateral aspect.
- c. Ora Cervices. Vaginal aspect.

tween gonads of the same animal and no correlation was indicated between ovarian and body weights. A highly significant difference was noted in gonad weights between individuals; this observation may be the result of differences in estrous cycle phases between animals.

Although little is known of the basic reproductive pattern of this species, the individuals included here exhibited closed vaginal openings (presumably indicative of diestrus). More adequate understanding of the physiology of reproduction of *C. lanigera* must be acquired before any meaningful conclusions can be drawn from the large differences in ovarian weights between individual animals.

Endocrine organs

Adrenal

The most striking feature of chinchilla adrenals is the large discrepancy in both size and shape between right and left glands of the same individual. The left adrenal lies antero-medial to the left kidney and is cylindrical in shape. The right adrenal is triangular in shape and lies near the longitudinal axis of the body, directly above the posterior vena cava at the point of entrance of the latter into the liver. A well-defined left adrenal vein fuses with the left renal vein prior to the emptying of the latter into the vena cava; the right adrenal is so intimately associated with the vena cava that no right adrenal vein is discernable.

Adrenals of 62 animals were weighed. The average weight of the left adrenal was 54.3 mg and that of the right adrenal was 36.4 mg. Adrenal weights appear to be influenced by neither body weight nor age. Five animals (two females and three males) were not included in table 1 because their adrenals were 8 to 10 times heavier than those of the 62 individuals assumed to be representative of the normal population. The most extreme case was a female whose right and left adrenal glands weighed 602.0 and 527.1 mg, respectively.

Pituitary

The chinchilla pituitary varies in shape from oblong to nearly round, in dorsal aspect. The neurohypophysis lies directly on top of the main bulk of the gland, along the long axis of the adenohypophysis. When fresh, the former is of the same whitish color as the cortical medulla; the anterior lobe is a deep reddish-gray. The dura mater is exceptionally

TABLE 1
Organ weights in C. lanigera

ORGAN	N	MEAN $\pm$ S.E.	t	P
		mg		
Testis	76	2599.8 $\pm$ 88.2		
Right	38	2547.1	0.845	> 0.05
Left	38	2652.5		
Ovary	44	25.7 $\pm$ 1.49		
Right	22	26.7	0.999	> 0.05
Left	22	24.6		
Adrenal	122			
Right	60	36.4 $\pm$ 1.53	9.12	< 0.01
Left	62	54.3 $\pm$ 2.08		
Pituitary	66			
Fresh weight				
Male	43	14.7 $\pm$ 0.93	3.73	< 0.01
Female	23	18.5 $\pm$ 0.92		
Per cent body weight				
Male	43	3.46 $\pm$ 0.21	2.46	< 0.05
Female	23	4.02 $\pm$ 0.22		

tough, tightly binding the pituitary into the shallow sella turcica, which in this species is a flat, oblong depression in the floor of the braincase. The sphenothmoid complex is considerably reduced, imparting the appearance of an inverted ribcage to the brainpan.

Males and females exhibit a significant difference in the weights of their pituitary glands. In common with other mammalian species, the pituitaries of females are heavier than

those of males; this proved to be true both in terms of gland weight and gland weight per cent body weight. The average weight of male glands was 14.7 mg and of female glands was 18.5 mg. Male hypophyses represent 3.46 and female hypophyses 4.02 mg % body weight, respectively.

Body weights

Adult

The average weight of 42 adult male chinchillas was 422.3 gm, and of 22 females 464.9 gm. These findings support the observations of commercial breeders that females are larger than males. No correlation was found between age and body weight in either sex.

Juvenile

The body weights of 14 chinchillas (six males and eight females) were taken at intervals from birth to 76 days of age. Birth weights of 5 additional young animals have been included although they failed to survive the duration of this study. These 19 animals had an average birth weight of 39.7 gm, showing no difference between the weights of males and females.

An average constant weight increase of 3.04 gm per day (fig. 4) was maintained over the period of measurement by individuals of both sexes.

TABLE 2
Body weights in C. lanigera

CATEGORY	N	MEAN $\pm$ S.E.	t	P
		gm		
Adult male	42	422.3 $\pm$ 9.23	3.92	< 0.01
Adult female	22	464.9 $\pm$ 11.9		
Birth	19	39.7 $\pm$ 1.39		
Male	9	41.0		
Female	10	38.7	0.856	> 0.05

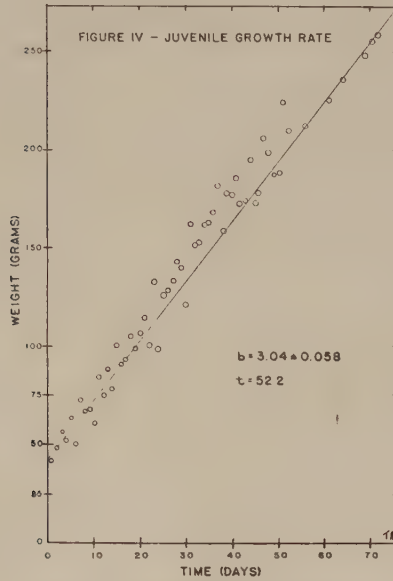


Fig. 4 Juvenile Growth Rates.

SUMMARY

1. The gross anatomy of the male and female genital tracts of the mature chinchilla (*C. lanigera* Bennett) has been described and diagrammed.

2. Gonad, adrenal, pituitary, and body weights of adult chinchillas have been tabulated and discussed.

3. Weights of animals for the first two and one-half months post-partum have been recorded and a growth curve constructed.

TERATOGENIC ACTIVITY OF SEVERAL AZO DYES CHEMICALLY RELATED TO TRYPAN BLUE¹

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THIRTY-FOUR FIGURES

Gillman, Gilbert and Gillman ('48) showed that injection of an aqueous solution of trypan blue into female rats before and during pregnancy caused maldevelopment in the offspring. It has been confirmed that treatment only during pregnancy caused abnormal development by Hogan, O'Dell and Whitley ('50) in the rat; by Murakami ('52), Hamburg ('52 and '54), and Waddington and Carter ('52) in the mouse; and by Harm ('54) in the rabbit. In a refinement of their original experiment Gillman et al. ('51) found that malformations occurred rarely when injection was given only before pregnancy, occurred in 8.6% of offspring when given only during pregnancy, but occurred in 32.4% of young when given both before and during pregnancy. Recently Wilson ('54b) reported that a related dye, azo blue, was equally as effective as trypan blue in producing developmental anomalies, but it was later necessary to withdraw this claim (Wilson, '54c) when it was discovered that the sample of dye used had been mislabelled by the manufacturer, and was in reality trypan blue. Despite this error, the experiment demonstrated that approximately 50% of offspring were regularly malformed after intensive treatment with trypan blue on the 7th, 8th and 9th days of pregnancy.

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The simplicity of the procedure and the high frequency of malformations make the use of trypan blue a promising method for studying the mechanism of action of a teratogenic agent. An initial effort in that direction is the present attempt to establish a relationship between teratogenic activity and chemical structure, by testing a series of azo dyes more or less chemically similar to trypan blue.

PROCEDURE

Pregnancy in 219 albino rats of the Hamilton Farms Strain (descendant from Wistar stock) was dated from the morning on which sperm were found in the vaginal smear, the conceptus being considered *one day of age* 24 hours after the finding of sperm. Laparotomy was performed on the 7th day to verify pregnancy and count the number of implantation sites. Immediately following the operation the animals were injected subcutaneously with 1 cm³ of 1% aqueous solution of one of the dyes being tested. Similar injections were given on the 8th and 9th days of pregnancy, making three injections comprising a total dose of 30 mg dry weight of the substance used. Since all were healthy, young, virgin females ranging between 200 and 250 gm, the dosage was not graded relative to body weight.

Pregnancy was allowed to continue until the 20th day when the mothers were killed in order to count the offspring *in utero*. The young were removed, examined for external malformations, weighed and preserved. Two from each litter were placed in 95% alcohol for clearing by the Schultz-Dawson method to reveal skeletal structure. The remainder were fixed in Bouin's fluid for subsequent dissection or histologic serial sectioning. Dissection consisted of making 1-2 mm slices transversely with a razor blade through the head and trunk regions. These slices were examined under a dissecting microscope and dismembered where necessary to show the gross structure of the brain, eye, palate, major arteries, heart, lungs, diaphragm and the intra-abdominal portions of the digestive, urinary and reproductive systems.

Dissection was routinely performed on the head of all young and on the bodies of those exhibiting head or external abnormality. Selected animals with external abnormality were prepared by routine methods for histologic study. Representative placentae from each litter and samples of maternal lung, liver, spleen, adrenal, kidney and lymph node were fixed in Bouin's fluid and prepared for histologic study by lightly staining with hematoxylin and eosin.

Most of the compounds tested³ were commercially available dyes of the quality used in biological staining. Those not soluble in distilled water to the extent of 1% were recrystallized from acid solution so as to yield crystals sufficiently small to permit injection as a suspension¹.

OBSERVATIONS

Fourteen azo dyes and o-tolidine, an important ingredient in the manufacture of several of these dyes, were tested for teratogenic activity by the above standard procedure. The first 4 dyes listed in table 1 are considered to possess teratogenic activity demonstrable by the methods used. Although malformation was also recorded after 3 others, Congo red, Erie garnet, and Niagara blue 3RD, in these cases the abnormality was limited to a moderate degree of hydrocephalus, a condition known to occur spontaneously in a low incidence (probably less than 1%) in the strain of rats used. This and the fact that in each case the abnormal animals came from a single litter are the basis for questioning the teratogenic potency of these three dyes. A low incidence of malformed offspring also followed treatment with Niagara blue 4B and Niagara sky blue 6B, but after these the malformations were not limited to hydrocephalus and in each case the defective young came from three different mothers.

³ Trypan blue, Niagara blue 4B, Niagara sky blue 6B, Congo red 4B, chlorazol black E, benzopurpurin 4B, Erie garnet B, and o-tolidine were obtained from Matheson, Coleman and Bell, Inc., Norwood, Ohio. Erie violet 2B, dianil blue 2R, Niagara blue 3RD, vital red, and acid milling red were obtained from National Aniline Division of Allied Chemical and Dye Corp., New York, N. Y. Evans blue was obtained from Distillation Products Industries, Rochester, N. Y. Azo blue was prepared by the Dr. G. Grüber & Company.

The two dyes most active in causing malformations, trypan blue and Evans blue, also caused higher rates of intrauterine death in pregnancies continuing to the 20th day than did the other substances that caused little or no malformation. A crude correlation thus seems to exist between the rates of malformation and mortality among fetuses in surviving pregnancies. This could be accounted for in part by assuming that certain malformations had lethal effects during prenatal life and that such lethal anomalies occurred in proportion with the overall incidence of malformation. In other words, the higher the malformation rate the higher the prenatal mortality rate. Other factors were evidently involved, however, because prenatal death exceeded the normal range of 8 to 12% after some of the dyes that produced no malformations.

The data in table 1 show that no correlation existed between malformation in surviving litters, and the number of pregnancies failing to reach the 20th day. This appears to mean that the effects of a dye on the survival of the mother and on the continuation of her pregnancy were independent of the effects of the dye on the developmental processes of the young. For example, vital red and chlorazol black frequently caused maternal death or interruption of pregnancy but never resulted in malformation of the young in pregnancies that continued to the 20th day. Conversely, treatment with trypan blue, Niagara blue 4B or Niagara sky blue 6B only occasionally resulted in early termination of pregnancy but did cause developmental abnormality in surviving offspring. It is noteworthy that another type of agent, maternal hemorrhagic anemia, when applied with sufficient severity to cause maternal death or early termination of pregnancy in a high percentage of cases, also failed to have an appreciable effect on development of the young in surviving litters (Wilson, '53).

Types and frequency of malformations: The types of malformations produced by the less active dyes Evans blue, Niagara blue 4B and Niagara sky blue 6B, were the same as the commoner types produced by trypan blue. Since the

frequency of occurrence, as well as the variety of types, however, was more limited after the less active dyes (table 2), it is probable that the various organs potentially susceptible

TABLE 1

Effects on gestation and the offspring of azo dyes injected on the 7th, 8th, and 9th days of pregnancy

DYE	TOTAL NUMBER MOTHERS	PREGNANCIES TERMINATING BEFORE 20TH DAY		PREGNANCIES CONTINUING TO THE 20TH DAY			
		% ma- ternal death	% complete resorp- tion	% of all preg- nancies	Number implan- tations	% of embryos resorbed	% of sur- vivors mal- formed
Trypan blue	45	4	16	80	407	44	49
Evans blue	20	10	25	65	162	23	14
Niagara blue 4B	15	7	13	80	126	15	4
Niagara sky blue 6B	15	0	20	80	145	8	3
Congo red 4B	20	30	15	55	123	15	1 ¹
Vital red	10	10	70	20	17	12	0
Acid milling red RN	9	11	11	78	78	13	0
Chlorazol black E	16	19	25	56	91	21	0
Benzopurpurin 4B	10	20	10	70	73	19	0
Dianil blue 2R	10	10	0	90	102	10	0
Erie violet 2B	13	0	8	92	148	11	0
Erie garnet B	10	0	0	100	112	7	2 ¹
Niagara blue 3 RD	10	0	0	100	115	11	1 ¹
O-tolidine	10	0	0	100	109	8	0
Azo blue	6	0	0	100	57	7	0

¹ Hydrocephalus affecting one or two animals in a single litter, not attributed to action of the dyes for reasons described in text.

TABLE 2

Frequency of malformations in various organs and systems after different dyes

	TOTAL NUMBER LIVING YOUNG	% OF YOUNG MAL- FORMED	% OF TOTAL LIVING YOUNG SHOWING DEFECTS IN VARIOUS ORGANS AND SYSTEMS				
			Brain	Eye	Cardio- vascular	Vertebral column	Other ¹
Trypan blue	228	49	33	10	11	17	12
Evans blue	124	14	13	2	1		1
Niagara blue 4B	107	4	4		1		
Niagara sky blue 6B	134	3	2	1			

¹ Specified in text.

to the teratogenic action of the azo dyes possess a threshold of sensitivity. Certain organs, such as the brain, would appear to have a low threshold and thereby occasionally subject to teratogenic action by the dyes of lowest potency while other organs only rarely affected even by trypan blue might be regarded as having a high threshold. There is no reason

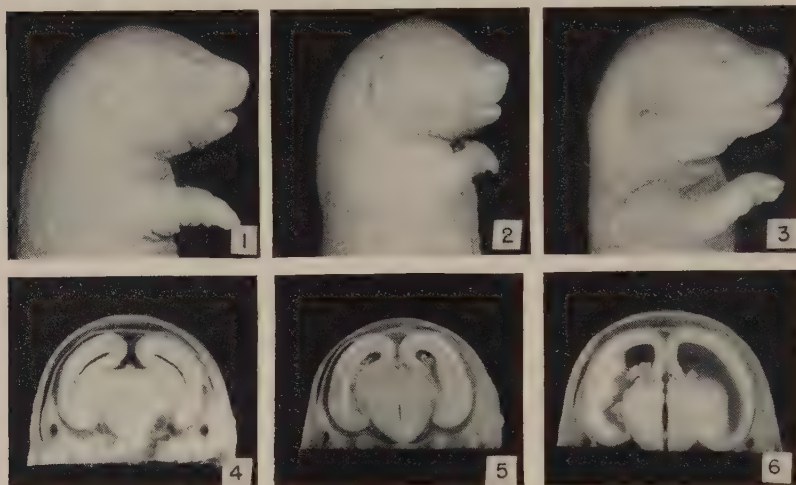


Fig. 1 Normal 20-day rat fetus.

Fig. 2 Mild hydrocephalus in 20-day fetus from mother injected with trypan blue on the 7th, 8th, and 9th days of pregnancy.

Fig. 3 Advanced hydrocephalus in fetus from mother injected with trypan blue.

Fig. 4 Transverse slice made with razor blade through brain of normal 20-day fetus.

Fig. 5 Slice through mildly hydrocephalic brain of 20-day fetus from treated mother.

Fig. 6 Slice through extremely hydrocephalic brain. The third as well as the lateral ventricles are distended.

to doubt that the mechanism of action is similar in the case of all 4 dyes; in fact, unpublished data on the use of larger doses than employed in the present experiment support this belief. It was found that raising the dose of the low-potency dyes increased the incidence of the commoner anomalies and resulted also in the appearance of certain types ordinarily seen only after treatment with trypan blue.

The brain was the organ most sensitive to the teratogenic action of azo dyes (table 2). Approximately 80% of brain defects after trypan blue and all after the other dyes were in the form of internal hydrocephalus (figs. 1-6). In the few hydrocephalic animals thus far examined histologically the mesencephalic aqueduct was either obliterated or reduced in calibre (figs. 7, 8). The cause of reduction was not apparent; no evidence of undue proliferation in the vicinity of the



Fig. 7 Section through mesencephalic aqueduct in normal 20-day rat fetus. $\times 23$.

Fig. 8 Section through aqueduct of a 20-day fetus from a treated mother. This animal had moderate hydrocephalus in the lateral and third ventricles and in addition a small meningoencephalocele. $\times 23$.

aqueduct was found. The ependymal lining was retained although sometimes was little more than a cord of atrophic epithelial cells. Other less frequent abnormalities of the brain were meningocele, meningoencephalocele and exencephaly (figs. 9-11).

Ocular anomalies, although less frequent (table 2), were often associated with brain abnormalities. Anophthalmia accounted for about half of the ocular defects, was rarely bilateral, and occurred more often on the left than the right

side (figs. 12, 13, 19, 20). Simple microphthalmia and occasional frank malformations such as retinal coloboma comprised the remaining developmental abnormalities (figs. 14-18). Probably not developmental but degenerative in nature was cataract of the lens (fig. 16), which was observed in a number of animals with no other defect of the eye. The exact incidence of cataracts is not known since the condition was recognizable only in histologic preparations and relatively few fetuses have yet been sectioned. Cataracts are known to occur spontaneously in an occasional animal of this strain, but all of the active dyes caused an unquestioned increase in incidence.

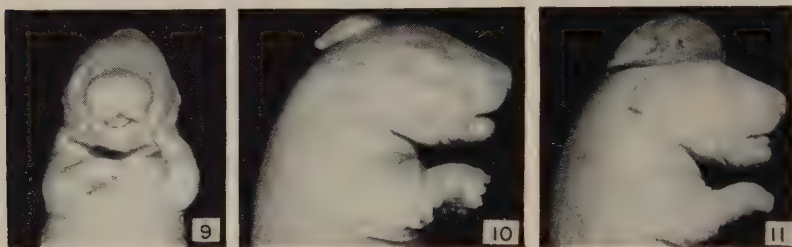


Fig. 9 Meningocele in 20-day rat fetus from injected mother.

Fig. 10 Meningoencephalocele in 20-day fetus from injected mother.

Fig. 11 Exencephaly in 20-day fetus from injected mother.

Cardiovascular anomalies occurred primarily after treatment with trypan blue but solitary instances were found after other dyes (table 2). The defects were about equally distributed between the aortic-arch arteries and the heart, both being abnormal in the same animal in approximately one-third of those exhibiting some cardiovascular anomaly. The aortic-arch anomalies were of several types; transposition of aorta and pulmonary trunk (figs. 21, 22), right-sided arch of aorta (figs. 23, 24), double arch of aorta, absence of ductus arteriosus, and truncus arteriosus communis (in descending order of incidence). The cardiac anomalies always consisted of some degree of defect in the interventricular septum (figs. 25, 26) and occasionally involved other structures, as was

noted in one case each of *cor biloculare* (fig. 27) and of *atrioventricular valvulis communis*.

The vertebral column was frequently rendered anomalous by treatment with trypan blue (table 2) and, when doses larger than 1% were employed (unpublished data) was occasionally affected by treatment with Evans blue. This category does not include such defects as *spina bifida*, which are considered to involve the vertebral column only secondarily. It consists of a group of malformations recognizable externally by absence or shortening of the tail and/or shortening of the trunk (figs. 28-30), but the underlying vertebral defects were best visualized in cleared, alizarin-stained specimens in which it was apparent that varying numbers of lumbar, sacral and caudal vertebrae were either rudimentary or absent (figs. 31-33). In extreme cases the entire sacrum was absent and the two ilia displaced medially, sometimes appearing to be in direct contact. The latter defect resulted in considerable reduction of the internal capacity of the pelvic cavity, associated with which were varying degrees of confluence, stenosis and atresia of the genitourinary and digestive tracts. Imperforate anus and absence of genital tubercle and genitourinary orifices were occasionally noted. These various midline defects in the caudal end of the body suggest a tendency toward the *sirenomelous* type of malformation, which typically involves fusion of the hind extremities, together with medial compression of the pelvic skeleton and absence of certain pelvic viscera. The fact that one perfect specimen of *sirenomelus* was obtained (fig. 34) strengthens the view that the other animals with vertebral and visceral deficiencies in the caudal region represent lesser degrees of this condition.

Other types of malformations were individually less frequent than the preceding, but numerous enough collectively to account for an appreciable percentage of the total abnormality (table 2). Excluding defects of the genitourinary and digestive tracts mentioned in connection with the vertebral anomalies, the additional types were: 5 cases of *gastroschisis*,

4 of short snout, 4 of clubfoot, three of spina bifida, and one each of meningomyelocele, diaphragmatic hernia, situs inversus totalis, and micromelia.

The types and percentage incidence of malformations observed here are generally comparable to those reported by Gillman, et al. ('51), with a few notable exceptions. The earlier authors did not find cardiovascular anomalies, which constituted about 22% of all malformations in the present experiment, but it may be that they did not undertake the dissection or serial sectioning necessary to demonstrate such defects. The previous investigators noted a somewhat higher incidence of spina bifida than was found here. These and other less obvious differences in results may be attributable to differences of procedure and in the strain of rats used.

Accumulation of the dyes in maternal and fetal tissues. Of the 15 substances tested (table 1) only trypan blue was accumulated in recognizable form by the maternal reticulo-endothelial system. After injection of this dye macrophages in the maternal lungs, liver, spleen, lymph node and general connective tissue were found to contain moderate numbers of dye-colored granules. Also the maternal kidney tubules, particularly the proximal convoluted segment, were heavily impregnated with dye-colored granules of varying sizes. When the dosage of the dyes possessing limited teratogenic activity was increased from the standard 1% to a 2 or 3% solution, these were also found to be accumulated in small to moderate amounts by maternal macrophages and slightly by the maternal kidney tubules (unpublished data). The fetuses did not contain visible forms of any of the dyes, hence, if the dyes crossed the placenta, they did so in minute amounts and were not accumulated in any tissue sufficiently to be grossly or histologically visible. The dyes used are not subject to reversible oxidation-reduction shifts, hence do not exist in a leuco form.

The chorio-allantoic part of the placenta failed to accumulate any of the dyes in appreciable amounts, although trypan blue was collected in scattered granules in an oc-

casional trophoblastic giant cell. The epithelium of the yolk sac placenta, however, contained large numbers of granules of trypan blue and lesser amounts of Evans blue, Niagara blue 4B and Niagara sky blue 6B. It is curious that these dyes were accumulated in this accessory placental structure in amounts roughly proportional to their teratogenic activity.

COMMENT

Four diazo dyes have been shown to possess teratogenic activity. Attempts to relate this activity to the chemical structure of the molecule, however, have met with limited success. (See Conn, *Biological Stains*, '53, for structural formulae of the dyes.) It is clear that the activity exhibited strongly by trypan blue and to a lesser degree by Evans blue, Niagara blue 4 B and Niagara sky blue 6 B is not shared by diazo dyes generally. Nine dyes of this class were shown here to lack such activity (table 1) and Gillman et al. ('51) reported that three others, Sudan IV, Bismark brown and trypan red, likewise failed to cause maldevelopment. Chlo-razol black, a triazo dye, was also shown to be ineffective. The active differed from the inactive substances in no specific way, although certain general differences were noted. The active dyes have the highest molecular weights (961 to 993) of any dyes thus far tested except trypan red. They possess molecules in which the central portion, that between the two azo linkages, consists of either o-tolidine (trypan blue and Evans blue) or o-dianisidine (Niagara 4B and Niagara 6B). Some of the inactive compounds also have o-tolidine in the central portion of the molecule, but these have fewer sulfonic acid, hydroxyl and amine substitutions on the naphthalene side rings and in several instances have these asymmetrically arranged around the two naphthalene rings. O-tolidine alone, without azo linkages and naphthalene side-rings, was totally ineffective. It is hoped that isomers and other compounds closely related to the active substances will soon be available for further study on the chemical configuration responsible for the teratogenic activity.

The site of primary action, whether in the embryo or in the mother, also remains obscure. The fact that the dyes have not been shown to cross the placenta introduces the possibility that they affect the maternal organism first, causing metabolic or other disturbance which in turn act adversely on the developing embryo. Gillman et al. have shown that chronic treatment of the mature female rat with trypan blue results in anemia, increased sedimentation of erythrocytes, hyalinization of renal glomeruli, fatty degeneration and other changes in the liver, and enlargement of the adrenals, spleen and lymph nodes. No one of these can be cited as teratogenic in itself and, in fact, maternal anemia (Wilson, '53) and liver damage (Wilson, '54a) have been found separately to have little or no effect on developmental processes of the embryo. Nevertheless, the mothers are known to experience more or less severe physiologic derangements, and it remains to be shown that collectively or in some combination these may not alter development of the embryo.

Speculations on the manner and site of action of these dyes must also take into account the puzzling fact that frequently some fetuses in a litter were highly abnormal while others in the same litter were normal, as far as could be determined. With trypan blue, where approximately half of the surviving fetuses were abnormal, it was the rule to find normal and abnormal offspring randomly interspersed in the same uterus. Gross and histologic comparisons of the placentae and membranes of normal and abnormal animals have yielded nothing that would account for these differences in reaction. Since it is reasonable to believe that the stimulus reaching all fetuses is the same, it would appear that the fetuses vary in their susceptibility to the stimulus. The genetic or other intrinsic basis for such variation, however, is not understood.

SUMMARY

The teratogenic (anomaly producing) activity of trypan blue and 13 other chemically related dyes was tested by in-

jecting these substances in 1% aqueous solution into pregnant rats on the 7th, 8th and 9th days of pregnancy.

Trypan blue was the most potent, causing malformations in 49% of living offspring from treated mothers. Somewhat less effective were: Evans blue which caused abnormality in 14%, Niagara blue 4B which caused anomalies in 4%, and Niagara sky blue 6B which caused malformations in 3% of offspring. The remaining dyes showed no activity.

The organs and regions most frequently affected were, in descending order, the brain, vertebral column, heart and major arteries, and eye; but several other structures were occasionally affected. Aside from frequency, the pattern of abnormality appeared to be the same after all 4 of the active dyes.

Only trypan blue was accumulated in macrophages of various maternal tissues and by the uriniferous tubules of the maternal kidney. All active dyes were collected to some extent by the yolk sac epithelium but by no other part of the placenta. No dyes were found in any fetal tissue.

It is not yet possible to relate the activity to a particular chemical configuration within the molecule, but the active compounds were found to have certain features of molecular structure in common. The site and mode of action of these dyes has not been determined.

ACKNOWLEDGMENT

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PLATES

PLATE 1

EXPLANATION OF FIGURES

- 12 Transverse section made with razor blade through eyes of normal fetus.
- 13 Anophthalmia on left side, mild microphthalmia on right, in 20-day fetus from treated mother.
- 14 Malformation consisting of retinal coloboma and rudimentary lens in left eye. Right eye is structurally normal.
- 15 Section through eye with cataract of the lens, from 20-day fetus from treated mother. This defect is not considered developmental but degenerative and is known to occur in a low incidence of the offspring of untreated females of the strain used. $\times 27$.
- 16 Section of microphthalmic eye which also shows eversion and coloboma of the retina and cataract of the lens. $\times 27$.
- 17 Abnormality of the retina in which the usual cellular layers are disrupted or replaced by irregular masses of what appear to be nerve fibers. Figure 15 shows a lesser degree of this condition. $\times 90$.
- 18 Section through a normal eye from a 20-day fetus. $\times 27$.
- 19 Normal 20-day fetus showing the usual external bulges caused by the eyeballs, even though the lids are closed.
- 20 Anophthalmia as evidenced by lack of bulging over the orbital region in a 20-day fetus from a treated mother.

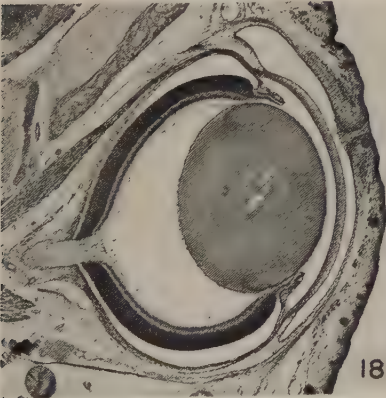
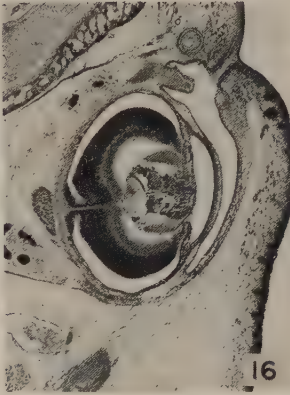
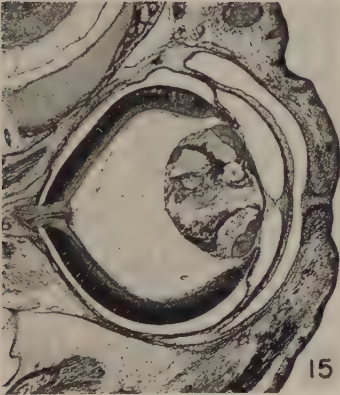


PLATE 2

EXPLANATION OF FIGURES

- 21 Section through the heart of a normal 20-day fetus. The pulmonary trunk (pul. tk.) is seen to arise from the right ventricle and proceed toward the descending aorta by spiralling from right to left about the ascending aorta. $\times 23$.
- 22 Transposition of the great vessels in a 20-day fetus from a treated mother. The pulmonary trunk arises from the left ventricle while the ascending aorta arises from the right ventricle and both take a roughly parallel course toward the descending aorta. $\times 23$.
- 23 Slice made with razor blade through the upper thoracic region of a normal 20-day fetus. The ductus arteriosus is seen to pass to the left of the trachea and esophagus to reach the descending aorta.
- 24 Right-sided arch and ductus in a 20-day fetus from a treated mother. The ductus passes to the right of the trachea and esophagus to reach the descending aorta which lies to the right of the esophagus, indicating that the arch of the aorta also passed to the right side.
- 25 Slice through the heart of a normal 20-day fetus.
- 26 Interventricular septal defect in a 20-day fetus from a treated mother. The right and left ventricles are seen to communicate through a large foramen in the interventricular septum.
- 27 Cor biloculare or two-chambered heart in a 20-day fetus from a treated mother. There was virtually no indication of the interatrial and interventricular septa and the atrioventricular canal remained undivided.

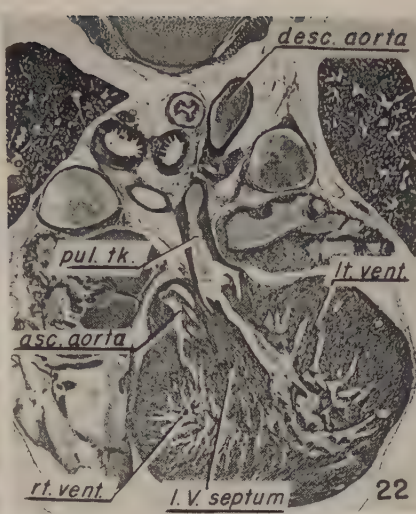
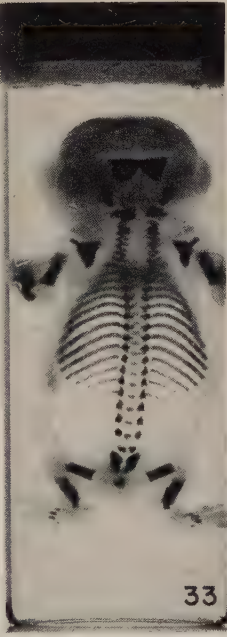
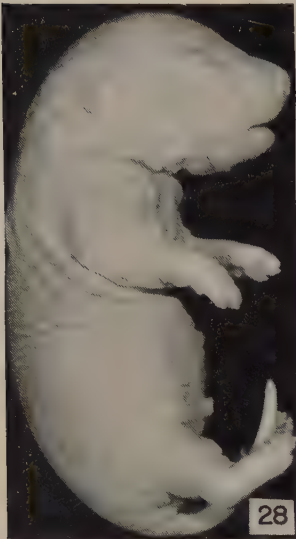


PLATE 3

EXPLANATION OF FIGURES

- 28 Normal 20-day fetus.
- 29 Short trunk in a 20-day fetus from a treated mother. The tail is missing and undoubtedly several lumbar and sacral vertebrae are also absent or rudimentary. Anal and genital orifices are often absent in such animals. In this specimen the legs are held at a peculiar angle, but it has not been determined whether this represents an additional anomaly or is only secondary to the vertebral and pelvic abnormality.
- 30 Short trunk complicated by spina bifida in a 20-day fetus from a treated mother. These two malformations are only occasionally associated and are considered to have different developmental origins. This animal also has clubfoot and absence of the tail.
- 31 Cleared, normal 20-day rat fetus. Note that there are 6 lumbar vertebrae, 5 sacral vertebrae, and 3 ossified vertebrae in the caudal region.
- 32 Vertebral abnormalities in a 20-day fetus from a treated mother. All vertebrae caudal to the third lumbar are either rudimentary or absent.
- 33 Vertebral and pelvic abnormality in a 20-day fetus from a treated mother. The lower lumbar, sacral and caudal vertebrae are absent and the paired bones of the pelvis are either closely approximated or in actual contact.
- 34 Perfect example of sirenomelus, a 20-day rat fetus from a treated mother. The tail and anal and genital orifices are absent. Serial sections revealed absence of most pelvic viscera and midline fusion of bones of the pelvis and hind extremity.



AN UNUSUAL VARIATION IN ORIGIN OF THE HEPATIC AND SPLENIC ARTERIES

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TWO FIGURES

Variations relative to the origins of one or more of the visceral branches of the abdominal aorta have been described by numerous investigators, including Lipschutz ('17), Eaton ('17), Kiss ('26), Adachi ('28), Browne ('40), Michels ('42, '51, '53), and others.

In view of the attention that variations in these arteries has received, it might have been supposed that the majority of important deviations would have been found and recorded. However, in the course of recent routine dissections, one unusual case of anomalous arrangements has been encountered. The distribution of arteries in this case bears little or no resemblance to any description previously reported so far as could be determined by an exhaustive search of the literature. Therefore, it seemed of sufficient interest to merit the following account.

OBSERVATIONS

The following variations were found in a white, male cadaver, 66 years of age. The celiac axis appeared to be absent (figs. 1 and 2). Nevertheless, the left gastric artery originated in the region which tends to approximate the mean location of the celiac axis as reported by Anson and McVay ('36), George ('35), and Cauldwell and Anson ('43). The origin of the superior mesenteric artery was found to

be more or less in its normal location approximately 1 cm inferior to the left gastric artery and opposite the inferior one-third of lumbar vertebra 1. It will be noted that the first branch of the superior mesenteric artery was the in-

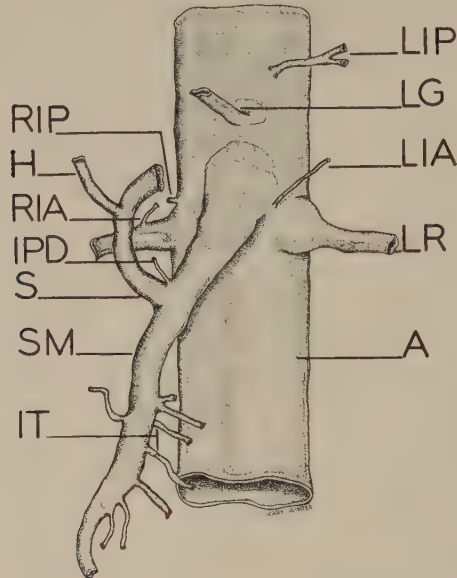


Fig. 1 The arrangement of some of the vessels arising directly or indirectly from the abdominal aorta in a male cadaver 66 years of age.

ABBREVIATIONS

A, Aorta
 GD, Gastroduodenal
 H, Hepatic
 IPD, Inferior pancreaticoduodenal
 IT, Intestinal branches
 LG, Left gastric
 LGE, Left gastroepiploic
 LIA, Left inferior adrenal
 LIP, Left inferior phrenic

RG, Right gastric
 RGE, Right gastroepiploic
 RIA, Right inferior adrenal
 RIP, Right inferior phrenic
 S, Lienohepatic trunk
 SM, Superior mesenteric
 SPD, Superior pancreaticoduodenal
 SV, Splenic vein

ferior pancreaticoduodenal artery. The next branch of the superior mesenteric artery appeared to be the splenic artery with the common hepatic being derived from the latter. In the present case this arrangement of the above arteries could be considered as a lienomesenteric trunk with an aber-

rant replaced common hepatic artery originating from the splenic artery or it might indicate the formation of a lienohepatic trunk, since both the splenic and hepatic arteries appear as replaced structures. Michels ('42) has reported one case in his series of 100 cases where the splenic artery originated from the superior mesenteric. The above investigator has also reported two cases of the hepatic artery

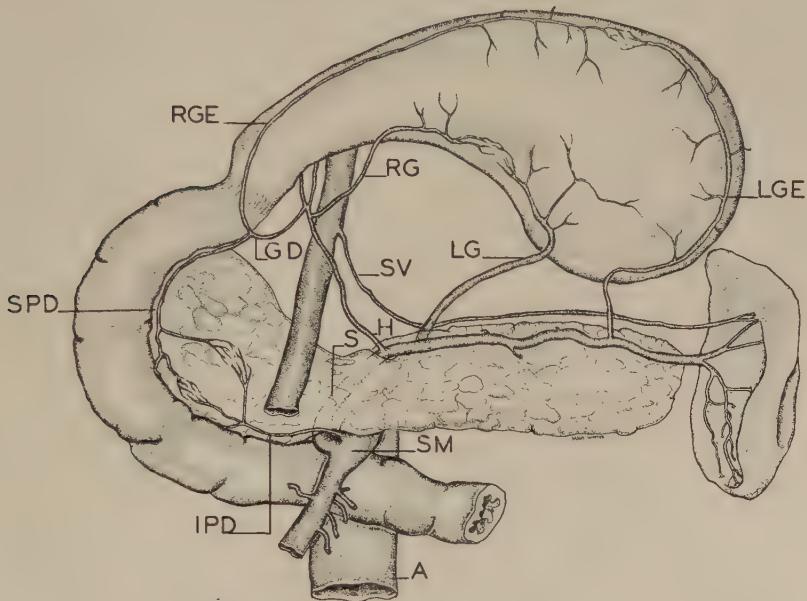


Fig. 2 The superior mesenteric artery and some of its branches; the stomach has been elevated and the peritoneum removed. The splenic vein has been elevated from its posterior pancreatic position and the portal vein drawn anteriorly.

arising from the superior mesenteric artery. The common hepatic artery, with an origin from the superior mesenteric artery, has also been reported as occurring in 22 of 500 cases examined by Daseler et al. ('47).

In the present case the left gastric artery originates in the region where the celiac axis normally occurs. It may indicate the replacement of the remaining portion of the celiac axis to by what has been described as the so-called

"truncus coeliaco-mesenterica." This trunk is apparently a normal condition in the Anura, some of the Reptilia and also some of the Mammalia. Tandler ('04) suggested that the formation of this trunk was the result of the persistence of the large primitive ventral anastomosis between the early segmental coeliac axis and the superior mesenteric groups. The major portion of the trunk is the superior mesenteric and, since this is the stronger vessel, it in this way assumes the branches which usually arise from the coeliac axis. The presence of a partial persistence of this longitudinal anastomosis might then explain the occurrence of the origin of the splenic, the common hepatic or both from the superior mesenteric artery. Bremer ('04) suggested that an extrinsic factor in the form of some developing sympathetic fibers might entwine the celiac root to alter the growth and thus the final position of that vessel in the chick. Broman ('08) explained the common trunk formation by the active outgrowth of a wandering root from the celiac which might attach itself to the superior mesenteric artery rather than the aorta.

The inferior phrenic and suprarenal arteries were also observed in this specimen. It is obvious (fig. 1) that the origin of the above vessels does not conform to the usual textbook description. However, as reported by Pick and Anson ('40, '40b) and Cauldwell and Anson ('43), these arteries are highly variable and their specific topography in the present specimen was not unusual. It is sufficient to state that the inferior phrenic arteries each originated from the aorta but the vertebral levels of each were quite different. The arterial supply to the suprarenals was derived from branches of the inferior phrenic and the renal artery on the right side while on the left the inferior phrenic and an artery derived from the aorta in common with the left renal supplied the gland.

During the course of dissection of this specimen, it was found that the splenic vein (fig. 2) was much smaller in caliber than its companion artery. It was also noted that the majority of the gastric and pancreatic veins drained directly into the portal or inferior mesenteric veins. The splenic

vein drained the spleen for the most part and entered more superiorly into the portal vein than the inferior mesenteric.

SUMMARY

Several anomalous arrangements of visceral branches of the abdominal aorta were discovered in one of the bodies during the course of routine laboratory dissections in gross anatomy. The left gastric artery originated in the usual site of the celiac artery. The splenic and common hepatic arteries were replaced and took origin from a common trunk off of the superior mesenteric artery. The possibility of a lienohepatic trunk or a replaced celiaco-mesenteric trunk are discussed.

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ASH WEIGHT OF HUMAN SKELETONS IN PER CENT OF THEIR DRY, FAT-FREE WEIGHT ¹

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ONE FIGURE

There is an increasing literature pertaining to the composition of bone of both laboratory animals and man. In animal studies for which the entire skeleton was available usually only selected parts of a given bone have been analyzed. Examination of samples of human bones have necessarily had to suffice in most studies, although there are reports on analyses of one or more entire bones from a given skeleton and of samples of the entire skeleton.

Experimental studies have indicated that although length and volume of the femur in growing rats were not appreciably altered within a range of calcium levels in the diet, variations in the width of the marrow cavity resulting from variations in the width of the compact bone were produced (Bell, Cuthbertson and Orr, '41). In another study on the femur and tibia of adult female rats, bone was resorbed during lactation and replaced during the next gestation. Although different parts of the bones contributed widely different amounts during the resorption phase, all regions were involved. When the calcium in the diet was nearly adequate the cancellous bone was more subject to resorption than was the compact bone, and when the calcium in the diet was very inadequate the compact bone was affected in almost the same proportion as the cancellous bone (Ellinger, Duckworth and

¹ This investigation has been supported by a research grant from Altrusa International.

Dalgarno, '52). Analyses of samples of the bones in these two studies showed a *practically* constant relationship between the organic and inorganic constituents.

In the first study the exception to this constant relationship occurred when the calcium intake was very low. The authors wrote, "The diminution of the percentage of Ca in these circumstances is most likely due to the greater proportion of periosteal and fibrous tissue on these very thin bones. It was extremely difficult to remove this fibrous matter without damage or fracture, and thus these very fragile bones may have been less thoroughly cleaned than the sturdier ones. We are inclined to believe that the actual composition of the bony material was constant throughout the experiment."

In the second study, analysis of the bones from those rats which had had the least calcium intake showed a reduction in the percentage ash of 3-5%. Again, these authors doubt that this percentage reduction is due to disproportion between the losses of organic and inorganic constituents. They suggest that it is, rather, the result of remnants of non-osseous organic matter in the interstices of the bone which could not be removed by their cleaning method. With resorption of bone the weight of true bone decreases and interstices containing non-osseous organic material become larger. Since the non-osseous organic material could not be removed completely, whatever remained would be an additional factor in the weight of the bone before ashing and thus, the percentage ash weight would be reduced.

In a recent review of the literature on the physiology of calcium metabolism, Nicolaysen, Eeg-Larsen and Malm ('53) presented a tabulation of the scanty data they were able to trace pertaining to weight and calcium content of adult human skeletons. This tabulation includes data from studies of 1 American, 13 European and 15 Ceylonese skeletons. Some additional data have been found but no report in which the entire skeleton has been analyzed either *in toto* or bone by bone. The American skeleton of a 35 year old, White, male cadaver was studied by Mitchell, Hamilton, Steggerda and

Bean in 1945 at the University of Illinois. Parenthetically, it may be noted that their plan was to analyze a number of cadavers in the age range of 20 to 50 years and in satisfactory nutritive condition. The second skeleton in the series was reported on by Forbes, Cooper and Mitchell in 1953. It came from a 46 year old, White, male cadaver. Neither of the bodies from which the skeletons came had been embalmed. Three individual bones of each skeleton (left tibia, ulna, and 9th or 10th rib) were analyzed separately as well as samples of the total skeletons. The weights of the ash in per cent of the dry, fat-free weight of the bones were given. Although comparable data of the two studies were similar, the statement was made in the second study that "These data support the thesis that analysis of a single bone is not likely to give a true estimate of the composition of the entire skeleton of an animal."

Since analyses of bones, both animal and human, have been restricted largely to selected long limb bones, it is timely to extend the study to each of the long limb bones from a given body. In order to understand the skeleton as a whole it is necessary to include each bone of the body. Incidentally data will become available with which to test the thesis of the workers at the University of Illinois. The possibility of masking the relationship between the organic and inorganic components should be considered. The purpose of this paper is twofold: first, to compare the ash weight in per cent of the dry, fat-free weight of long bones which are from limbs prepared differently before skeletonization and which have been subjected to different fat solvents in the process of reducing them to a fat-free state; and second, to determine the percentage ash weight of each bone and its relationship to the ash weight of the whole skeleton from a series of adult cadavers of different ages.

MATERIALS AND METHODS

All the cadavers from which the bones came were White males except one which was a Negro male. The bones studied

include the long limb bones (femur, tibia, fibula, humerus, radius and ulna) from 10 unembalmed cadavers, and complete skeletons from 7 cadavers which have been prepared differently. Thus, there are long limb bones from 17 cadavers, and all other bones of the skeletons from 7 of these cadavers. Table 1 summarized details of the source and preparation of the bones studied.

Embalming was accomplished either with 10% formalin or with a combination of glycerine, alcohol, and carbolic acid crystals (referred to subsequently as GAC). The exceptions are cadavers OS-343 and OS-354 which were embalmed before delivery to this laboratory. There was evidence that the embalming fluid contained formalin as well as other constituents. These cadavers are an exception, also, because they were stored in a 3% carbolic acid solution for two years or more before skeletonization; the other embalmed cadavers were so stored for no more than a few weeks, if at all.

After removing most of the soft parts by dissection, the remaining soft parts were macerated by immersion in water kept just under the boiling point by means of an automatic thermostat. The cleaned and air-dried bones were subjected to benzol vapors for approximately 17 hours in order to "degrease" them. The methods of preparation up to this point follow exactly the methods of preparation of the skeletons in the Terry Collection (Trotter, '54).

The bones were dried in an oven at 70°C. for 7 days or longer. The weights were constant after the 4th or 5th day. Immediately after removal from the oven the weights were taken on a platform balance to the nearest 0.1 gm. At this time the bones were fat-free to the same degree as those in the Terry Collection. A number of the bones were then subjected to another degreasing process consisting of immersion in acetone for 24 hours. Tests of longer periods with several changes to fresh acetone did not result in reducing the bone weight. The acetone treatment was followed by drying and weighing. This weight is considered to be the weight of dry, fat-free bones. It is recognized that the term, dry fat-free

TABLE 1
Source and preparation of bones

Number	CADAVER DATA		Embalming	FAT REMOVER	
	Age	Cause of death		Benzol for all	
OS-840	62	Bleeding esophageal varices	None	+ acetone for left bones	
OS-841	68	Lobar pneumonia	None	+ acetone for left bones	
OS-842	53	Lobar pneumonia	None	+ acetone for left bones	
OS-844	80	Accident	None	+ acetone for left bones	
OS-846	41	Chronic myocarditis	None	+ acetone for left bones	
OS-845	67	Chronic myocarditis	None	+ acetone for right bones	
OS-847	49	Cirrhosis of liver	None	+ acetone for right bones	
OS-848	70	Chronic myocarditis	None	+ acetone for right bones	
OS-850	50	Hanging	None	+ acetone for right bones	
OS-851	53	Gun shot in head	None	+ acetone for right bones	
C-402	89	Arterio-sclerotic heart disease; Pulmonary tuberculosis	Right limbs with GAC	+ acetone for all bones	
OS-874	72	Unknown	Right limbs with GAC	+ acetone for all bones	
OS-881	60	Coronary thrombosis	Head and Neck with GAC	+ acetone for all bones	
OS-882	45	Accident	Right limbs with formalin; Head and Neck with GAC	+ acetone for all bones	
OS-354	73	Chronic myocarditis	Unknown	+ acetone for all bones	
43-4,111	56	Paresis; Urinary infection	GAC		
OS-343	65	Coronary occlusion	Unknown		

bone in this study includes in addition to the osseous organic and inorganic constituents some remains of non-osseous organic material, since the bones were brought to the dry, fat-free state in the whole condition.

Ashing was carried out in a muffle furnace at 600°C. Each bone was ashed separately except for the cranium², movable vertebrae, hand bones, foot bones and right and left ribs. These were ashed as named. All were put in the furnace intact with the following exceptions: the femurs, which were sawed transversely in half because the furnace could not accommodate their length; the crania which had the calvariae separated; some of the ribs which had been sawed. The weight of the ash is recorded as a percentage of the weight of the dry, fat-free bone before ashing.

RESULTS

Long limb bones

The ash weights of the long limb bones in per cent of their dry, fat-free weights according to age, preparation and side of the body are presented in table 2.

Of these 17 sets of long limb bones, the set from cadaver OS-343 will be omitted from the following comparisons because the percentage ash weights of the bones are at such variance with those from other cadavers. It should be noted, however, that the bones were not greasy either in appearance or to the touch, nor did they appear to be fragile.

The average percentage ash weights of the long limb bones according to the method of embalming show only small differences which are not consistent for any one of the methods. Although the number of limbs in each category is small, it is believed that these differences are not attributable to differences in embalming.

An increase in the percentage ash weight of a bone after immersion in acetone is found for the 93 bones, when this

² The word *cranium* is used throughout this paper to denote skull without mandible.

TABLE 2

Ash weights of long limb bones in per cent of their dry fat-free weights according to age and preparation. For each cadaver the values for the right bones are given in the first line, those for the left bones in the second line. The columns headed I are percentage values for bones which were degreased only with benzol; columns headed II are percentage values for bones which were degreased first with benzol and then with acetone.

NUMBER	AGE	EMBALMING	FEMUR		TIBIA		FIBULA		HUMERUS		RADIUS		ULNA	
			I	II	I	II	I	II	I	II	I	II	I	II
OS-846	41	None	62.3	66.2	63.8	65.9	63.8	67.6	64.5	66.4	65.4	67.1	64.9	68.0
OS-822	45	Formalin None	65.3	66.5	65.6	66.2	65.8	67.4	65.4	66.3	66.4	67.2	66.0	67.1
OS-847	49	None	65.5	66.3	66.5	67.2	66.2	67.0	66.9	67.6	65.3	67.0	62.3	67.2
OS-850	50	None	65.7	66.6	65.1	66.3	65.6	67.7	68.1	68.5	65.8	68.1	63.8	64.7
OS-842	53	None	62.4	65.2	62.9	65.8	63.6	65.7	64.1	65.8	64.1	65.4	63.1	66.5
OS-851	53	None	65.4	66.5	65.4	66.5	65.5	67.5	65.0	66.1	65.9	68.1	66.1	67.8
43-4,111	56	GAC	66.6	66.6	66.0	66.0	68.6	63.3	63.3	64.9	64.9	64.2	64.2	64.0
OS-881	60	None	66.7	68.0	66.7	67.2	67.4	67.6	67.7	67.6	67.4	68.4	68.1	68.2
OS-840	62	None	63.4	67.2	64.1	66.7	65.2	66.3	62.9	65.7	62.0	66.2	65.1	66.7
OS-343	65	Unknown	55.6	57.7	54.1	57.7	49.5	53.5	53.5	49.3	49.3	47.4	47.4	48.5
OS-845	67	None	66.1	66.8	66.2	67.4	64.5	67.2	65.2	66.2	63.6	67.2	65.3	67.8
OS-841	68	None	63.5	66.4	63.4	66.1	64.5	67.4	65.0	66.8	63.9	67.6	65.7	66.1
OS-848	70	None	67.2	68.1	67.4	68.5	64.4	67.4	68.0	68.9	67.8	69.5	68.5	69.6
OS-874	72	GAC None	58.4	65.2	61.6	65.6	65.3	66.2	65.1	65.9	65.3	65.6	66.1	66.4
OS-354	73	Unknown	64.6	67.1	64.9	65.7	67.0	67.9	63.9	64.8	65.5	66.1	65.5	66.2
OS-844	80	None	62.1	67.4	62.1	67.7	62.6	68.8	61.3	68.2	62.7	68.3	63.0	68.8
C-402	89	GAC None	62.7	65.2	61.2	63.4	54.0	62.8	58.7	63.2	60.3	63.2	52.2	64.4
			64.4	65.0	66.3	66.9	65.6	65.8	63.1	65.0	63.6	65.3	66.0	66.3
					65.2	65.5	63.7	64.2	64.6	65.0	66.2	66.2	66.1	66.2

figure is compared with the weight after exposure to benzol vapors only (table 2). The amount of increase varies from bone to bone but the variation is less within one set of long limb bones than between sets from different cadavers. For the following bones, the average increase in the percentage ash weight is: humeri, 1.5%; tibiae, 1.6%; radii, 1.8%; femora, 1.9%; ulnae, 2.7%; fibulae, 3.7%.

The greater percentage ash weight of the right or left bone of any given pair after the same method of degreasing shows no dominance of one side of the body over the other for either upper or lower limbs (table 2). It should be noted also that the percentage ash weights of the two bones of a pair show less difference after degreasing in acetone than in benzol vapors.

When the averages of the percentage ash weights of the bones from cadavers 60 years and less are compared with the averages of bones from cadavers over 60 years of age, it is found that the younger group shows a slightly higher average than does the older group (table 3). This difference is present for bones degreased with benzol vapors, as well as for bones degreased also by immersion in acetone. The only exception to this is the tibia for which the average percentage ash weights after the second degreasing are the same in the younger and older groups.

*Total skeletons*³

The ash weights in per cent of the dry, fat-free weight of the remaining bones from 7 cadavers in order of age are tabulated in table 4.

The percentage ash weights of these complete skeletons, including the Negro, are quite uniform with a range of only 2.1% from 64.8% to 66.9%, except for one, OS-343. This skeleton has a percentage ash weight of 54.5% — from 10 to 12% lower than any one of the other 6. The treatment it

³ The auditory ossicles, the hyoid bone, and the coccyx unless fused to the sacrum are not included.

received before skeletonization is different from that of the others, excepting OS-354. These exceptions were noted above.

The percentage ash weights of most of the individual bones comprising each skeleton vary less than 5% from the total percentage ash weight. The bones of each skeleton with percentage ash weights of 5%, or more, below that of the complete skeleton are for:

- skeletons OS-881 and OS-874, none;
- skeleton C-402, left patella;
- skeleton OS-882, sacrum, right clavicle and right hip bone;
- skeleton 43-4,111, sternum, sacrum and left hip bone;
- skeleton OS-354, sternum, sacrum, vertebrae, and ribs;
- skeleton OS-343, sacrum, sternum, hip bones, vertebrae, right clavicle, ribs, ulnae, radii, and right fibula.

TABLE 3

Average percentage ash weights of bones of younger and older groups according to the methods of degreasing

BONE	BENZOL		BENZOL AND ACETONE	
	Years 41-60	Years 61-89	Years 41-60	Years 61-89
Femur	64.8 (14)	64.0 (13)	66.7 (9)	66.4 (10)
Tibia	65.1 (14)	64.3 (14)	66.5 (9)	66.5 (11)
Fibula	64.8 (13)	62.8 (13)	67.1 (8)	66.6 (10)
Humerus	65.0 (14)	64.1 (14)	67.1 (9)	66.2 (11)
Radius	65.6 (14)	64.4 (14)	67.4 (9)	66.7 (11)
Ulna	64.7 (14)	63.9 (14)	67.4 (9)	67.0 (11)

The figures in parentheses indicate the number of individual bones contributing to the average.

Thus, in two skeletons there is no individual bone with a percentage ash weight which is as much as 5% below the percentage ash weight of the complete skeleton and, in another skeleton there is only one bone, the left patella, with an ash weight below this value. In two skeletons there are three bones in each with low percentage ash weights; sacrum and hip are two bones, whereas the third is not the same bone in the two skeletons. In one, it is the right clavicle and in the other the sternum. In skeleton OS-354, the sternum and sacrum are each approximately 10% below the complete

TABLE 4

Ash weight in per cent of dry, fat-free weight of bones of the skeleton (exclusive of long limb bones). Value of right side precedes value of left side

BONES	PERCENTAGE ASH WEIGHTS						
	OS-882 45 yrs.	43-4,111 56 yrs.	OS-881 60 yrs.	OS-343 65 yrs.	OS-874 72 yrs.	OS-354 73 yrs.	C-402 89 yrs.
Hip	58.9 62.1	61.9 59.4	65.3 65.5	38.8 38.3	65.2 64.1	61.1 60.4	64.4 64.4
Patella	63.6 67.5	64.4 65.6	67.5 67.2	49.6 53.3	64.8 64.8	66.1 66.0	61.8 49.2
Foot	64.5 65.7	65.6 65.2	66.3 65.1	57.8 59.3	63.3 65.6	66.8 67.3	62.4 66.3
Clavicle	58.8 64.7	64.0 64.2	68.1 66.8	43.3 50.4	64.7 65.6		63.3 64.9
Scapula	62.5 64.3	66.3 67.1	66.4 66.4	47.6 46.7	63.8 65.4	62.8 63.6	65.5 65.1
Hand	65.5 65.3	61.6 61.6	66.3 63.7	58.1 56.0	63.6 64.5	67.2 66.5	64.2 64.6
Cranium (teeth)	66.2 (16)	66.3 (none)	68.2 (none)	68.6 (14)	66.6 (none)	68.4 (3)	67.5 (none)
Mandible (teeth)	69.7 (13)	69.0 (6)	68.2 (none)	71.3 (12)	68.5 (none)	69.9 (3)	69.8 (none)
Vertebrae							
cervical	62.4		66.6		65.7	61.1	64.6
thoracic	61.1		65.8		64.9	53.4	61.8
lumbar	62.9		65.2		66.3	54.8	63.0
Total	62.0	72.2	65.7	39.6	65.5	55.2	62.0
Sacrum	57.6	58.4	64.5	28.7	64.3	54.8	63.1
Sternum	61.8	55.2	65.2	31.0	64.0	54.2	62.7
Ribs	63.3 63.8	63.7 62.9	65.9 66.2	45.2 44.4	66.1 65.5	58.1 56.7	61.9 62.7
Total skeleton ¹	65.2	65.3	66.9	54.5	65.6	64.8	64.9

¹ The percentage ash weight of the total skeleton includes data of pertinent long bones listed in table 2. If two percentage ash weights according to methods of degreasing are available, it is the weight after immersion in acetone found in column II which is included to make the totals in this table. The bones of skeletons 43-4,111 and OS-343 were degreased only with benzol, all others were degreased with acetone as well.

skeleton; the vertebrae (excluding the cervical) are equally low; and, the left and right ribs, 6% and 8% respectively. In skeleton OS-343 of which the percentage ash weight of the complete skeleton is only 54.5%, many bones have a percentage ash weight well below this 5% level; the lowest are sacrum, sternum and both hip bones, in that order; then vertebrae, right clavicle, ribs, scapulae, ulnae, radii and right fibula. The only bones in this skeleton exceeding the total percentage ash weight are foot, hand, cranium, and mandible and three of the 12 long limb bones.

In 5 skeletons the bone with the highest percentage ash weight is the mandible. In the other two skeletons the mandible is exceeded in one, only by the vertebrae, and, in the other, only by the left radius. The presence of teeth does not seem to increase the percentage ash weight.

The differences between the highest and lowest percentage ash weights among the bones of each of these 7 skeletons are 4.7%, 5.2%, 12.1%, 16.5%, 17.0%, 20.6%, and 42.6%. The ages of the skeletons in corresponding order are: 60, 72, 45, 73, 56, 89 and 65 years.

DISCUSSION

Factors during life, such as age, nutrition, disease, etc., may affect the relative amounts of organic and inorganic matter in a dry, fat-free bone. Factors after death which may alter or mask the proportion of these constituents include decomposition of the body, effect of embalming fluid and method of storage and the processes involved in reducing the bone from its state in the cadaver to a dry, fat-free state. Presumably, conditions conducive to decomposition of the body from autolytic ferments are reduced to a minimum. The hospitals from which the cadavers came are all equipped with cold storage rooms and immediately upon delivery to this laboratory they are stored at 15° to 20°F. until embalmed or skeletonized.

There is slight evidence in this study based on the long limb bones that the percentage ash weight of bones of older

individuals is lower than that of younger individuals. The difference is so small, however, that a much larger series is needed in order to test its significance. No attempt was made to estimate the nutritional status of the cadavers, or to verify the stated cause of death.

Comparison of the percentage ash weights of bones from unembalmed and embalmed cadavers does not indicate a difference which can be attributed to the embalming process with the possible exception of one cadaver, OS-343. The other cadaver, OS-354, for which the formula of the embalming fluid is not known, was probably preserved with fluid of the same formula since they had been embalmed in the same morgue. In addition, both these cadavers were stored for a long period in a 3% solution of carbolic acid. The one which was stored for almost three years has a percentage ash weight of its total skeleton which is in keeping with those not so stored, whereas the one stored for just two years has a lower percentage ash weight by 19%. It must be mentioned, also, that the ash weights are not exactly comparable from the standpoint of the methods of degreasing. As would be expected the higher percentage ash weight is found in skeleton OS-354 which had been degreased in both benzol and acetone, while the lower percentage is found in OS-343 which had been degreased only with benzol. It is improbable that this unusually low percentage ash weight could be accounted for by this difference in the methods of degreasing (see table 2).

The reduction in weight of bones after immersion in acetone shows this to be a more efficient method of degreasing than exposure to benzol fumes according to the method which has been practiced in this laboratory. It is assumed that benzol used in a Soxhlet extractor, the method employed by Forbes, Cooper and Mitchell ('53), would have resulted in the same weight as did immersion in acetone. A possible source of error in the dry, fat-free bone weight as determined in this study is the remains of the non-osseous organic material which can be demonstrated easily in the medullary cavity when the femurs are sawed just before ashing. In small

samples of bone this has been removed by dissection (Baker, Butterworth and Langley, '46) and in larger samples by a constant-boiling aqueous mixture of ethylene diamine in a Soxhlet extractor (Williams and Irvine, Jr., '54). Neither method was applicable here, since it was desired to ash the whole bones. It is our opinion that the remains of the non-osseous organic material is a negligible contribution to the weight of the dry, fat-free bone in this study.

The literature provides very few figures of percentage ash weights of dry, fat-free human bones. Of those available, certain pertinent details of preparation of the body as well as of the bones may be missing. Nicholls and Nimalasuriya ('39) studied 15 male and 14 female adult skeletons which had been collected in the Department of Anatomy of Ceylon Medical College. "Ten samples of bones with contained marrow were taken from these skeletons. They were dried for three hours at 105°C. . . . The ash ranged from 64 to 68.4% of the dried samples." It would be interesting to know the ages of the cadavers, the methods of embalming, if any, and the skeletonizing process, the particular bones from which the samples were taken, and the size of the samples.

In both studies at the University of Illinois the skeletons were from unembalmed bodies of relatively young individuals who had died rather suddenly. In the 1945 study by Mitchell et al., the left tibia, ulna, and 9th rib, and in the 1953 study by Forbes, Cooper and Mitchell, the left tibia, ulna, and 10th rib were analyzed separately from the other bones. The second report gives more details concerning the method of preparation than does the first.

"Representative portions of the skeleton were comminuted as finely as possible with an iron mortar and pestle. . . .

"Moisture was determined on all the samples by drying them to constant weight in a vacuum oven at 70°. Either extraction of the dried samples was accomplished by the Soxhlet principle; a low boiling petroleum ether being used for 48 hours . . . Ash was determined with a muffle furnace at 600°."

Professor Mitchell writes (personal communication, '54) that "Ash weights of 1953 specimen were derived by obtaining the weighted average of ash (per cent) in left tibia, ulna and rib and 'remaining bones.' The latter fraction consisted of a selection of bones representative of the total remaining skeleton in so far as I could estimate it. The 1945 specimen was handled similarly except that all bones (after removal of left tibia, ulna and 9th rib) were ground in a bone mill, mixed well and sampled." The percentage ash weights of the dry, fat-free weights in these two studies are as follows:

YEAR	TIBIA	ULNA	RIB	TOTAL SKELETON
1945	66.34	64.00	57.54	56.67
1953	69.30	65.63	52.10	56.89

It may be seen in table 2 of the present study that the range of percentage ash weights of 20 tibiae (column II) is from 63.4% to 68.5% and of 20 ulnae (column II) 64.4% to 69.6% — percentages quite similar to those of the Illinois studies. Table 4 includes percentage ash weights for ribs and total skeletons. The ribs of each side were weighed together, rather than singly. In 5 skeletons the percentage ash weights of the ribs are higher than were found by Professor Mitchell and his collaborators, in skeleton OS-354 they are approximately the same and in skeleton OS-343 they are much lower. The percentage ash weights of 6 of the present total skeletons exceeded the Illinois totals by 9% or 10%, whereas that of skeleton OS-343 is approximately 2% less. It is difficult to understand how the percentage ash weights of the Illinois skeletons could have been as low as they were when the percentage ash weights of the individual bones were similar to their counterparts in skeletons of the present study with much higher totals.

It is recognized that the number of total skeletons and of additional long limb bones examined in this study is not sufficient to warrant statistical analysis. The findings do show, however, that the percentage ash weights of most individual bones of the same skeleton are quite uniform. The bones within a given skeleton most likely to present a percentage



Fig. 1 Thoracic vertebrae from the 45 year old skeleton, OS-882, on the left and from the 89 year old skeleton, C-402, on the right.

ash weight well below that of the total are those of the axial skeleton excluding cranium and mandible. This reduction may extend also into the bones of the limb girdles. It is not known whether the reduction is the result of an increase in the organic constituent or a decrease in the inorganic constituent of the dry, fat-free bone. Furthermore, the percentage ash weight cannot be predicted from the appearance or weight of the dry, fat-free bone. The thoracic vertebrae of the youngest and oldest skeletons studied may serve as an example (fig. 1). Except for the first and second, the thoracic vertebrae in the 89-year-old skeleton (C-402) were fused together in a solid bony mass, not only in the disk regions but also in the region of the supraspinous ligament. Many ribs were also fused to the vertebrae and had to be broken away. Doctor J. Albert Key, Professor of Orthopedic Surgery, Washington University, looked at this spine and made the diagnosis of ankylosing arthritis with secondary osteoporosis. No microscopic sections were made. On the other hand, the thoracic vertebrae of the 45-year-old skeleton (OS-882) were 12 individual bones of the text-book type, with no suggestion of fusion or even lipping. Data pertaining to these two thoracic spines are:

SKELETON	DRY, FAT-FREE WT.	ASH WT.	ASH WT.
	<i>gm</i>	<i>gm</i>	%
C-402	204.6	126.4	61.8
OS-882	123.8	75.6	61.1

The vertebrae which were atypical in appearance and morphology had both dry, fat-free weight and ash weight nearly twice that of the vertebrae with normal appearance, yet, the percentage ash weights of the two are practically identical.

The data provided by this study show that the percentage ash weight of dry, fat-free bone varies from skeleton to skeleton and, further, that the composition of one bone may not be a measure of the composition of other bones of the same skeleton. It is suggested that variation among percentage ash weights of the bones of a dry, fat-free skeleton

may be the result of a progressive change in composition which is initiated in one region and extended to other regions in an orderly sequence.

SUMMARY AND CONCLUSIONS

The long limb bones of 10 White male cadavers and the complete skeletons of 6 additional White male and one Negro male constitute the material studied. The age range of the cadavers is from 41 to 89 years. The cadavers or the limbs from which the bones came were treated differently before skeletonization. The effectiveness of two different methods for fat extraction was tested. From the dry, fat-free state, the bones were reduced to ash, and the weight of the ash was determined in per cent of the dry, fat-free weight.

There is no evidence to indicate that the state of the cadaver before skeletonization, either unembalmed or embalmed, affects the percentage ash weight of the dry, fat-free bones. The reduction of the weight of bones to constant weight by immersion in acetone for 24 hours indicates the greater efficiency of this method over exposure to benzol fumes for 17 hours.

The percentage ash weight is slightly higher in younger than in older long limb bones. This age difference does not apply, however, to the complete skeleton. The percentage ash weights show less variation among the bones of the free limbs and skulls than among the bones of the limb girdles and trunks. The appearance and weight of dry, fat-free bones is not indicative of their percentage ash weights. In 6 complete skeletons the total percentage ash weights varied from 64.8% to 66.9%; in one complete skeleton it was 54.5%.

Of the 7 complete skeletons there are two in which the percentage ash weights of all bones fall within a very close range of each other and, in each of the other 5 skeletons, bones are found with percentage ash weights that are 5% or more below that of the complete skeleton.

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INHIBITORS OF AMPHIBIAN DEVELOPMENT¹

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TWENTY-SEVEN FIGURES

In a developing embryo the processes of cell division, determination, morphogenesis and differentiation proceed in sequential fashion, but they also overlap and are mutually interdependent. It is of fundamental importance to disengage these processes experimentally by suppressing any one of them. This has been achieved to a degree by transplantation and explantation. Cell division occurs without differentiation in explanted ectoderm in the absence of the inductor. Conversely, organ primordia may show differentiation with partial suppression of growth *in vitro*. However, the integrating and regulating processes of the embryonic organism do not usually permit clear separation of these mutually interdependent processes in the intact organism. For this reason even chemical agents of diverse structure often produce similar effects. Some agents, however, do exhibit effects which are selective enough to permit study of individual developmental events.

In view of their structural similarity to the purines, the compounds employed here and in a previously reported work (Liedke, Engelman and Graff, '54) could conceivably play some role in nucleic acid metabolism. These compounds are somewhat similar in structure to the naturally occurring

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purines, adenine and guanine (fig. 1a). They also inhibit other biological systems such as *Tetrahymena geleii*, and *Escherichia coli* according to Engelman, Gillespie, Greer and Graff ('53), and some are mutagenic (S. Greer, personal communication).

Some of these compounds, the quinoxalines, particularly, inhibited *Rana pipiens* embryos in early cleavage stages. On the other hand, 4-methoxy-6-nitrobenzotriazole (MNB) (fig. 1c) caused arrests in later stages. The present study is concerned with an analysis of the action of 4-methoxy-6-nitrobenzotriazole (MNB) and 5-methoxy-7-nitroquinoxaline (MNQ) on *Rana pipiens* embryos treated in the tailbud stage.

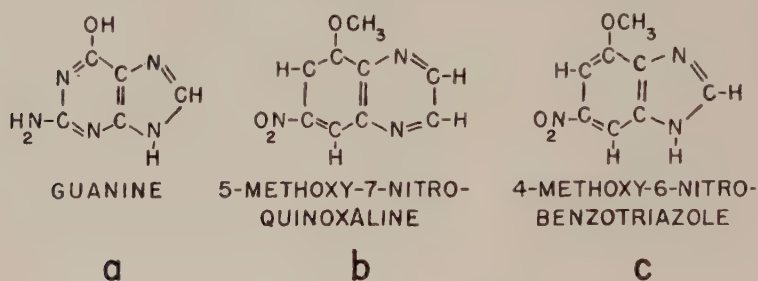


Fig. 1a-1c Comparison of chemical structure.

The selective response of early developmental stages (2-cell, blastula, gastrula and neurula) to the triazole and quinoxaline compounds made it appear desirable to test older embryos. The early tailbud stage 17/18 (Shumway, '40) was chosen since blastulae exposed to MNQ were always severely stunted in growth, and developed as dwarfed tailbud embryos showing extreme microcephaly, cellular enlargement and necrosis, while blastulae exposed to MNB were arrested as neurula or normal tailbud embryos without these cellular abnormalities. It was expected that this difference in action would be more apparent in developing structures and organs of the tailbud stage, depending on whether cell proliferation or differentiation predominates. If MNB were to interfere with differentiation more than with cell division one should detect such interference more readily in later stages of development.

MATERIAL AND METHODS

The embryos were obtained by the usual method of pituitary injection. For each experimental series embryos of the same age (stage 17/18) from the same female or egg batch were exposed to the two compounds (10 embryos per 20 ml solution in stendor dishes). Length of exposure varied (tables 1 and 2) and in some series embryos in the blastula stage were used for comparison. The solutions with concentrations ranging from 0.01 to 0.1 mg/ml were made up in 10% Ringer solution and adjusted to pH 6.7. After exposure the embryos were cleansed of the test compounds through repeated transfers to 10% Ringer solution. They continued development in finger bowls in 50 ml of 10% Ringer solution to which a little moss was added. The experiments were terminated after 6 days at 18–20°C. or after 7 days at 15–16°C. when the controls had reached stage 25. Abnormalities, survival and rate of progress in development were recorded using Shumway's table for stages in development.

For histological study some embryos were sacrificed at intervals of one to two days. They were fixed in Bouin's or Smith's fluid, sometimes in Carnoy's fixative. Serial sections were made and stained with borax carmine and 0.1% nigrosin in aqueous picric acid or with Harris' hematoxylin and erythrosin.

RESULTS

I. Comparison of Susceptibility

As tables 1 and 2 show, MNQ exerts a far stronger inhibitory influence on the younger embryo (blastula stage 8) than on the tailbud (stage 17/18). The effective dosage is always larger for the older stage, indicating an increase in resistance to MNQ with age. The reverse is true for MNB, namely, sensitivity increases with age of embryo.

The data in tables 1 and 2 were obtained by averaging the results on 8 different batches, which varied only slightly. Survival rate at maximum arrest by MNB was increased

when embryos after exposure were kept singly in separate stendor dishes instead of the usual procedure of 10 embryos per 50 ml of 10% Ringer solution in one fingerbowl. The isolated embryos developed very slowly and abnormally, nevertheless, even though survival increased.

The immediate arrest by MNB is most remarkable. The tailbud embryos remained at the same stage even after removal from MNB (table 2). Growth and advance to the next developmental stage 19 was usually observed one to two days after exposure, depending upon dosage, whereas the controls and the embryos exposed to MNQ had reached stage 21-22. Thereafter, development of the MNB treated embryos continued at the normal rate, but the initial arrest was never completely overcome. At the termination of the experiment, on the 6th or 7th day, when the controls were stage 25, the experimental animals were in stage 24 or younger (cf. fig. 13).

An immediate arrest was never obtained with MNQ, however. With this agent the first notable lag in development of the gills occurred on the 3rd day (stage 22). Abnormalities and delay in growth became apparent thereafter (4-6 days after exposure).

II. Comparison of morphological and histological effects

1. External morphology

(a) Embryos exposed to MNQ

The embryos appeared to be normal during the first 3-4 days following exposure to MNQ or until larval stage 21-22 was reached. After the fourth day, however, development of the gill filaments failed to proceed at the normal pace. In fact, the gills remained the same or even atrophied from the 4th to the 6th day (stage 22-25). Gill circulation was impaired and had ceased usually by the sixth day. The embryos were shorter than the controls. The operculum grew, but microcephaly and swelling was apparent (hydrops embryos). Muscular movement was also very poor. Apparently the circulatory system which is normally fully

established and functioning at stage 22-23 had become arrested, causing some of these anomalies at about that time.

(b) *Embryos exposed to MNB*

Embryos which had been exposed to MNB varied in appearance during the first three days depending on length of initial arrest at stage 18. In severe arrests caused by higher concentration or longer exposure, they were only at stage 21-22 on the sixth day when the controls had reached stage 25. These embryos apparently had never reached the stage at which the circulatory system is functionally established. In other embryos arrested gill development occurred after the 4th day. The external appearance of these embryos was then similar to embryos exposed to MNQ. Still others, at smaller dosage, retained normal appearance and circulation, although a slight delay in development was still apparent on the last day.

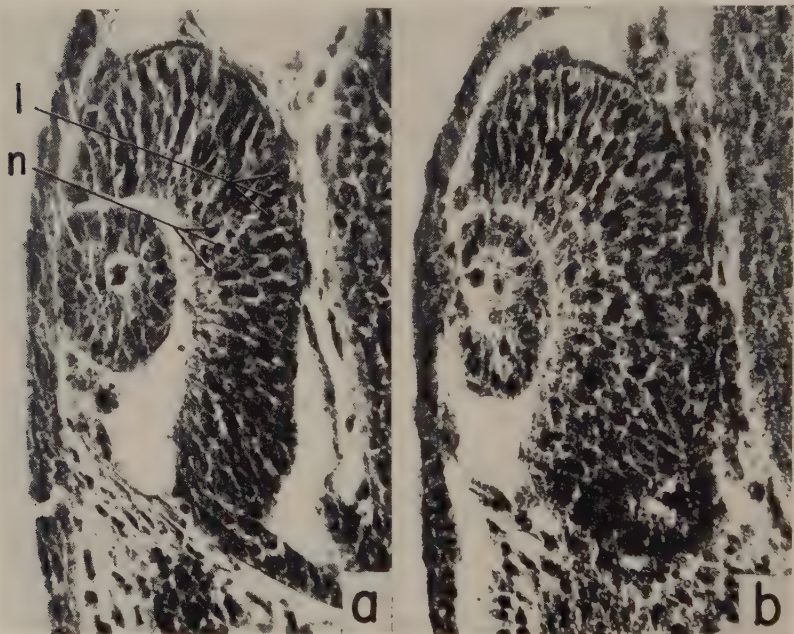
2. *Histology*

Destruction of the circulatory system causes certain abnormalities (Kemp, '53). It is possible, therefore, that the compounds are first inhibitory to the development of the circulatory system and subsequently cause the same secondary abnormalities which are found in embryos after excision of the heart. Only those embryonic arrests not found in cardiectomized embryos can be ascribed to the direct action of the compounds, MNB and MNQ. It appears then that abnormalities encountered during the first three days or before the circulation is fully established at stage 22-23, are directly caused by the compounds.

(a) *Embryos exposed to MNQ*

The development of the circulatory system to stage 21 was normal in all embryos which had been exposed to MNQ at stage 17-18. Stage 21 is reached after three days at 15°C., or after 2½ days at 18-20°C. At stage 21 the first primitive

blood elements have appeared in the heart, in the post-cardinal sinuses, the liver, gills and in some blood vessels. Yet, in spite of normal development of the circulatory system up to the third day, some organs, especially the eyes, are already abnormal even on the first and second day after treatment with MNQ (cf. figs. 2a, 2b, 4, 5, 7, 8).



Figs. 2a, 2b Comparison of experimental (MNQ) and control eyes.

(a) The eye of an embryo two days after continuous exposure to 0.05 mg/ml MNQ at stage 17/18. Note necrosis of early differentiating cells (n) in the center of the optic cup, while the marginal, still proliferating cells of the germinal layer are reduced in number and larger (l) (Carnoy, H. and E.). $\times 200$.

(b) Control eye (Carnoy, Borax Carmine). $\times 200$.

On the first day the cells are larger and stain lighter (fig. 4) than in the developing eye of controls (fig. 5), but a day later, or on the second day, the difference between normal and experimental embryos is more apparent (figs. 2a and 2b). It is seen that the total number of cells is reduced in the experimental optic cup and lens vesicle (fig. 2a) compared to

the control (fig. 2b). Necrotic cells in the center of the optic cup have released densely staining masses of chromatin. All other nuclei, proceeding from center to periphery, are enlarged. It is apparent that the centrally located nuclei were most severely affected and became necrotic by the second day. Embryologically these central nuclei are the oldest. At this stage of development they are no longer only mitotic but rather early differentiating cells, while the marginal cells in the germinal layer are younger, undifferentiated and still proliferating. MNQ, therefore, makes it possible to distinguish between early differentiating and proliferating cells. The former are most severely affected and break down early; the latter respond by nuclear enlargement, arrest at the interphase, and cessation of further cell division.

On the 4th day many of the enlarged interphase nuclei break down; at the same time new cells are mobilized in the outer germinal zone of the eye cup. These cells move centrally, their nuclei enlarge, and they also eventually break down. Orderly differentiation of the retinal layers of the eye therefore becomes impossible. Finally, on the 6th day (fig. 10) there is a marked cellular reduction and accumulation of cell debris in the eye cup. However, it should be noted that some lens cells were spared and could still differentiate (fig. 10). Figure 10 also shows that on the 6th day the germinal zone in the older eye is in the iris rim of the optic cup where enlarged nuclei are still present.

The brain responds in the same selective manner as the eye. On the third day enlarged nuclei and some necrotic cells are present, this process continuing to microcephaly. The ears fail to differentiate and remain as cystic sacs (cf. figs. 18 and 19). All mesenchymal cells are large and reduced in number; cartilage, muscle, in fact, all organs and structures, are reduced in size (fig. 26).

Since Kemp ('53) has shown that certain anomalies and reductions in size of organs or cell number occur in embryos in which the heart was excised, it is important to separate those secondary responses to circulatory disturbance from

the specific responses to the chemical agent. As was shown above, cellular abnormalities in the eye may be brought about before the circulatory system is established functionally. In fact, the selective cellular response to MNQ is repeated in the development of the circulatory system. At the time of exposure at stage 17-18 the circulatory system is non-existent; the heart is but a primordium of splanchnic mesenchyme cells, while the eye is already present as a definite structure, the optic vesicle, which invaginates during the next 24 hours to form the optic cup. MNQ does not interfere with cellular migration, proliferation, or morphogenic movements, for optic cup formation. The first necrotic cells were the early differentiating primitive neuroblasts seen in the center of the optic cup on the second day after exposure (fig. 2a). Neither does MNQ inhibit cell division and morphogenic movements necessary to establish the heart and circulatory system up to stage 21 (figs. 20 and 24). Under the experimental conditions this phase of morphogenesis lasted one day for the eye, but three days for the heart, depending on temperature or concentration of MNQ used. At higher concentrations or higher temperature (18-20°C.) the controls continued development to the next stage 22, while the experimental embryos remained arrested at the same stage 21. This is illustrated in figure 20 (experimental), figure 21 (controls of same age) and figure 24 (younger controls). It is seen that the hearts of the experimental embryos failed to grow after stage 21 and contain relatively few blood cells compared to the controls. It seems, therefore, that MNQ exerts its maximum effort on the developing circulatory system at a time when the primitive blood cells begin to differentiate and undergo rapid cell division. The primitive blood cells are not only larger and reduced in number (figs. 20, 21), but other structures like the wall of the heart, the pronephros, mesoderm, etc., possess larger and fewer cells. Some cells have already broken down. Even a few necrotic primitive blood cells are found on the third day. Cell deaths continue and cause microcephaly and partial atrophy of organs, such as

the heart on the 6th day (fig. 23). Few if any blood cells are left. Figure 14 shows some of these remaining blood cells. Some are of the same size, others are of larger size than the primitive blood cells of younger embryos at stage 21 (fig. 12). They are often filled with large yolk platelets which indicate metabolic interference with yolk utilization. Most nuclei are enlarged, some are lobular, and some cells contain as many as 4 nuclei. Since usually only two nucleoli could be identified, it is not possible to conclude that these are true polyploid cells which arose through entomitosis, but late prophase containing more than diploid number of chromosomes were found occasionally in these embryos. The separate nuclei of a single cell are also often connected by minute strands.

Similar delay in yolk absorption is found in cardiectomized frog embryos. Other abnormalities found with MNQ, namely edema, enlargement of pronephric tubules, failure of differentiation and elongation of the intestine, smaller size of most structures such as cartilage, muscle and nervous system, occur in cardiectomized embryos as well. But extensive cellular necrosis in the eyes and central nervous system were observed by Kemp ('53) only in 4 day post-operative cardiectomized embryos which must have been older than stage 25, at which stage the experiments with MNQ were terminated. Kemp did not report cystic ears, nuclear enlargement, or interphase arrest.

(b) *Embryos exposed to MNB*

Nuclear enlargement and necrosis were never found in embryos after exposure to this compound. In this respect they differ drastically from embryos exposed to MNQ. On the other hand, the most striking feature in these embryos was the overall stunting of growth and differentiation. Growth of all structures and organs was halted initially and then resumed, more or less successfully. The extent of these effects varied with concentration and length of exposure.

More severely arrested embryos showed various deficiencies in the development of the heart, the eye, and other structures. The eye shown in figure 9, for instance, is not abnormal, but has irregular cell groupings, an irregularity which is more pronounced in the lens. A few yolk-laden primitive blood elements are wedged ventrally between the retina and the lens. Apparently their migration into the eye cup and subsequent differentiation was arrested. The heart in this embryo barely managed to establish a small lumen (fig. 25) which contains only a few primitive blood cells. One embryo surviving for six days after 20 hours of exposure to 0.1 mg/ml MNB, a dosage which usually kills after one to two days (see table 1), has a most severely arrested heart with no lumen at all (fig. 22). Other structures in this embryo are also severely arrested. The forebrain has differentiated layers of grey and white matter, but the eyes are abnormal, too young and too small, compared to the brain (fig. 16). Even in this embryo which has a much younger and more severely arrested heart than ever found in embryos treated with MNQ, there are no necrotic areas in the eye or brain and no enlarged interphase nuclei indicating again that the circulatory system plays no role in this particular cellular response to MNQ. Neither is it a question of age of embryo, for younger eyes (figs. 2a and 4) already show this cellular response to MNQ.

DISCUSSION

Susceptibility to various agents usually decreases with age of the embryo. Inhibitors of respiratory metabolism such as cyanide and sodium azide are notable exceptions. The fact that susceptibility to MNQ decreases with age while the reverse is true for MNB, namely increased sensitivity of older more differentiated embryos (tables 1 and 2) is significant. The difference in sensitivity is the more remarkable in view of the fact that even one half hour of exposure to 0.1 mg/ml MNQ irreversibly arrests cleavage in the two-cell stage (Liedke, Engelman and Graff, '54) while at least 48

TABLE 1
Comparison of the development of *Rana pipiens* embryos exposed at blastula stage 8 and early tailbud stage 17/18 to 4-methoxy-6-nitrobenzotriazole (MNB) and 5-methoxy-7-nitroquinoline (MNQ)

COMPOUND CONTROLS	CONC.	HOURS EXPOSED	STAGE EXPOSED	DEVELOPMENTAL STAGES										
				12	13-14	15-16	17-18	19	20	21	22	23	24	25
MNB	0.1	24	8	12	12	13-14	14-15	14-15	14-15	14-15	14-15	14-15	14-15	14-15
MNQ	0.1	3-6	8	12	12	12	12	12	Died					
MNB	0.1	20	18				18	18	Died					
MNQ	0.1	20	18				18	19	20	21	22	23		
MNB	0.05	24	8	12	13	15	17	19	20	21	22	23-24		
MNQ	0.05	3	8	12	13	14-15	15-16	15-16	15-16	15-16	Died			
MNB	0.05	20	18				18	18	19	20	21	22		
MNQ	0.05	44	18				18	19	20	20	21	22		
MNB	0.025	**	8	12	12	12-13	12-13	Died						
MNQ	0.025	**	8	12	12	12-14	12-14	Died						
MNB	0.025	**	18				18	18	19	19	Died			
MNQ	0.025	**	18				18	19	20	21	22	23		
MNB	0.01	**	18				18	18	18	20	Died			
MNQ	0.01	**	18				18	19	20	21	22	23-24		

** Continuously.

hours or more are required in order to arrest development of embryos exposed in the tailbud stage. Exposing two-cell stages for as much as 30 hours to the same concentration of MNB still permits development to the blastula or early gastrula stage, before death ensues. But a much shorter exposure, 14–20 hours, arrests and kills tailbud embryos (stage 17–18). While it is true that MNB never arrests even

TABLE 2

Comparison of survival of Rana pipiens embryos exposed at tailbud stage 17/18 to 4-methoxy-6-nitrobenzotriazole (MNB) and 5-methoxy-7-nitroquinoxaline (MNQ)

COMPOUND	CONC.	HOURS EXPOSED	% SURVIVAL WHEN CONTROLS WERE AT STAGE:							
			18	19	20	21	22	23	24	25
MNB	<i>mg/ml</i>									
	0.1	10–14	100	100	100	100	100	97	91	58
		20	100	0 ¹						
	0.05	18–20	100	94	94	91	77	77	77	73
	0.05	24	100	30	0					
	0.025	20–44	100	100	100	100	100	100	100	100
	0.025	72	100	100	83	83	35	0		
	0.01	96	100	100	100	90	75	40	0	
MNQ	0.1	20	100	100	100	100	100	100	100	100
		22–24	100	94	91	91	78	78	71	63
	0.05	24	100	100	100	100	100	100	100	100
		72	100	100	100	95	50	0		
		120	100	100	100	100	95	95	5	

¹ Single embryos kept in individual dishes sometimes showed 30–50% survival in spite of extreme developmental arrest.

the most sensitive tailbud stage within half an hour, as does MNQ with the two-cell stage, selective permeability can hardly be a responsible factor. It is important to note that the histological response of the embryos differs markedly for the two compounds and that only the more differentiated stages, particularly neurulae and tailbuds are sensitive to MNB.

It must be concluded that the specific histological responses of the embryo to MNQ are not caused by defects in the circulatory system. In fact, these histological responses can

also be traced in the morphogenesis and differentiation of the circulatory system (figs. 20, 21, 23, 24, 27). It was shown that primitive blood cells are most sensitive to MNQ as they are to radiation also (Schjeide, Allen and Piccirillo, '52).

MNQ not only inhibits cell division, but has a differential effect on cell types. Thus early differentiating cells like the primitive neuroblasts or embryonic ganglion cells in the eye are affected most and break down first, while the younger, still proliferating marginal cells of the germinal layer enlarge and remain in the interphase (fig. 2a). Such a differential effect on cell types has been found with nitrogen mustard in amphibian and insect development and has been extensively analyzed by Bodenstein ('47, '54).

Radiation has a similar highly selective effect, destroying the primitive neuroblasts or early differentiating cells of rat and mouse embryos (Hicks, O'Brien and Williams, '54), of frog embryos (Rugh, '54), of Axolotl embryos (Brunst, '54). Other agents which are said to mimic the highly selective histological effects of radiation are triethylene melamine and some sulfhydryl reagents. But the "margin of safety" in radiation is larger, i.e. in irradiated brains of mice only early differentiating cells (primitive neuroblasts) are selectively destroyed, while older, more differentiated cells (neurons) and undifferentiated proliferated cells are spared. Proliferating cells are not spared by MNQ or by nitrogen mustard. Their nuclei enlarge and remain at the interphase. Rugh ('54) did not describe such degenerating giant cells in irradiated frog embryos. However, it is a well known fact that they do occur in both embryonic and adult tissues after irradiation. Degenerating giant cells in the eyes of irradiated Axolotl embryos were reported by Brunst ('54). Klein and Forssberg ('54) showed that irradiated ascites tumor cells grow to double size. These investigators demonstrated that DNA increases only slightly, while RNA and total N per cell increased at nearly the same rate as cell volume.

The cells of a younger embryo are larger than those of an older embryo. The question arises whether the enlarged

cells, seen in embryos after exposure to MNQ are actually enlarged cells or resulted merely from a block in cell division at a younger stage. No definite conclusion on this point can be drawn from the present work. It was found, however, that the nuclei in these enlarged cells of treated embryos (figs. 12, 14, 17), sometimes are even larger than those of younger embryos. The origin of these enlarged nuclei, many of which are lobular, fragmented, and possibly polynuclear, deserves further experimental analysis since the question of endomitosis is involved. Polyploid metaphases have not been found in these enlarged blood cells, but occasionally in enlarged mesenchyme and pigment cells. Poly- and heteroploid cells have been found in frog blastulae arrested by a conjugated carcinogenic hydrocarbon, 9,10-dimethyl-1,2-benzanthracene (Green, '54).

The high sensitivity of early cleavage stages to MNQ (Liedke, Engelman, and Graff, '54) is easily understandable because MNQ affects cell division. It is known (Sze, '53) that the rate of cell division is much greater in early than in later development. Obviously then, a block involving one or a few cells at an early stage must affect survival far more radically than later when many more cells are present and cellular damage can more easily be repaired. Therefore, in order of sensitivity, the histological response to MNQ is: cells of highest mitotic activity (i.e. undetermined cells of early cleavage stages) > early differentiating cells (i.e. primitive neuroblasts and primitive blood cells) > more differentiated cells (i.e. older neuroblasts and neurons). This explains why susceptibility is highest in early cleavage stages then decreases with age of embryo until the time when the circulatory system becomes functionally important at stage 22-23. Thus exposure to 0.1 mg/ml MNQ was fatal at the two-cell stage after one half hour, at stage 18 after 48 hours, at stage 22 after approximately three hours.

It seems, therefore, that the selective cellular response to MNQ depends on mitotic rate and on the state of cellular differentiation. Another selective but different cellular res-

ponse is found in fibrocyte cultures and amphibian embryos, where metaphases instead of interphases are arrested by some unsaturated steroids and some female sex hormones (Schenck, '50; Töndury and Cagianut, '51).

MNB effects are characterized by the absence of such a selective cellular response, and by heightened sensitivity of the embryo with increasing age and differentiation. Selective cellular damage never occurs in acute anoxia, or with cyanide and azide, or when insulin hypoglycemia is induced in embryos (Hicks, O'Brien and Williams, '54). In our previous study it was tentatively suggested that MNB interferes with embryonic differentiation, rather than with general cell division. A differentiating embryo has a mixed cellular population, the cells varying in degree of determination and in degree of early histogenesis. All these different cell types multiply at different rates, while processes of morphogenesis, induction and integration are taking place. Interference by MNB with any one of these various processes of embryonic differentiation could have caused delay in development and size reduction of the embryo shown in figure 13, or abnormalities in sensitive structures like the eye or heart shown in figures 16, 22, 25. If the long drawn-out inductive process in lens development is interrupted by explanting the early lens primordium, the explanted self-differentiating lens is very much smaller than control lenses (McKeehan, '54). Interruption of induction in ear development causes cystic ears (Detwiler and Van Dyke, '50). Not only interference with induction, but interference with cell division can prevent normal embryonic differentiation, i.e., the ears develop as cystic sacs in *Amblystoma* embryos after irradiation (Piatt and Ravenstos, '53) and in frog embryos after exposure to MNQ (fig. 19).

Until more factual evidence is obtained from isolated cell types and embryonic tissues, it is impossible to state which of these various processes occurring in embryonic differentiation were specifically affected by MNB. However, absence

of a selective histological response rules out primary interference with cell division.

A final explanation in terms of biochemical events is impossible. Both compounds are structurally related to purines and presumably could interfere with nucleic acid and protein synthesis. Bieber ('54) found that purine and pyrimidine analogues cause cyclic developmental inhibitions in frog embryos. The inhibitory effect of these antimetabolites could be modified when certain nucleic acid derivatives were added. Interference with sulfhydryl enzymes has been suggested for radiation and sulfhydryl reagents which cause radiomimetic cellular responses. Such unrelated substances as nitrogen mustard and MNQ also mimic the histological effects of radiation. It is, of course, possible that the action of all of these agents is indirect, i.e. causes intracellular damage which in turn releases a substance that inactivates a substrate or precursor.

SUMMARY

1. Embryos of *Rana pipiens* show fundamentally different stage-specific responses to MNQ and MNB. The sensitivity to MNQ is greatest in early developmental stages of highest mitotic rate and decreases in later stages. With MNB quite the reverse is true; early cleavage is not usually affected; the sensitivity of the embryo to MNB increases with age so that the compound is most effective in older, more differentiated embryonic stages, like the tailbud stage 17-18.

2. The morphological and histological responses of the embryo to these two compounds is markedly different. Early differentiating cells are most sensitive to MNQ while more differentiated cells and mitotic cells may be spared, depending on rate of cell division, or mitotic cells may become arrested at the interphase and then degenerate.

3. No selective cellular response occurred with MNB. MNB, rather, interrupts abruptly all further growth and differentiation. Some differentiating structures are more susceptible than others and normal recovery is possible, depending upon length of arrested period.

4. The specific responses and anomalies in development produced by MNQ and MNB were analysed and compared with those known to occur with other agents which affect the metabolism of the embryo. Some similarities and some distinct differences were found. A comparison with defects in development following cardiectomy showed that specific differences exist and that the compounds cause selective responses which are expressed even differently in the developing heart and circulatory system.

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PLATES

PLATE 1

EXPLANATION OF FIGURES

The eyes of embryos exposed to 0.1 mg/ml MNB for 10 hours (figs. 3, 6, 9) or to 0.1 mg/ml MNQ for 20 hours (figs. 4, 7, 10) and the eyes of control embryos (figs. 5, 8, 11) at one, three and 6 days after start of exposure at tailbud stage 17-18, temperature 15-16°C. The magnification of all photomicrographs is $\times 133$.

- 3 (MNB after one day). Development is blocked. The eye is still in the optic vesicle stage as the day before (cf. fig. 5, control eye).
- 4 (MNQ after one day). The delay in development is negligible, but nuclear enlargement and interphase arrest have started (cf. control eye, fig. 5, also fig. 2a).
- 5 (After one day). Control eye.
- 6 (MNB after three days). Development is still delayed but normal (cf. fig. 8).
- 7 (MNQ after three days). The eye and lens are smaller than those of controls (fig. 8). The nuclei are enlarged. More cells have become necrotic than on the second day (fig. 2a).
- 8 (After three days). Control eye.
- 9 (MNB after 6 days). The eye is normal except for some irregular cell groupings, and smaller size. Differentiation of a few rods and cones and the ganglionic layer started (cf. control eye fig. 11). Note the dense mass of yolk laden arrested primitive blood cells containing some necrotic elements (dense staining), ventral to the lens.
- 10 (MNQ after 6 days). The eye shows advanced necrosis and loss of cells. Note that enlarged nuclei are still present in the germinal zone at the presumptive iris margin. Some lens cells were also spared and continued histogenesis.
- 11 (After 6 days). Control eye showing differentiation of retinal layers.

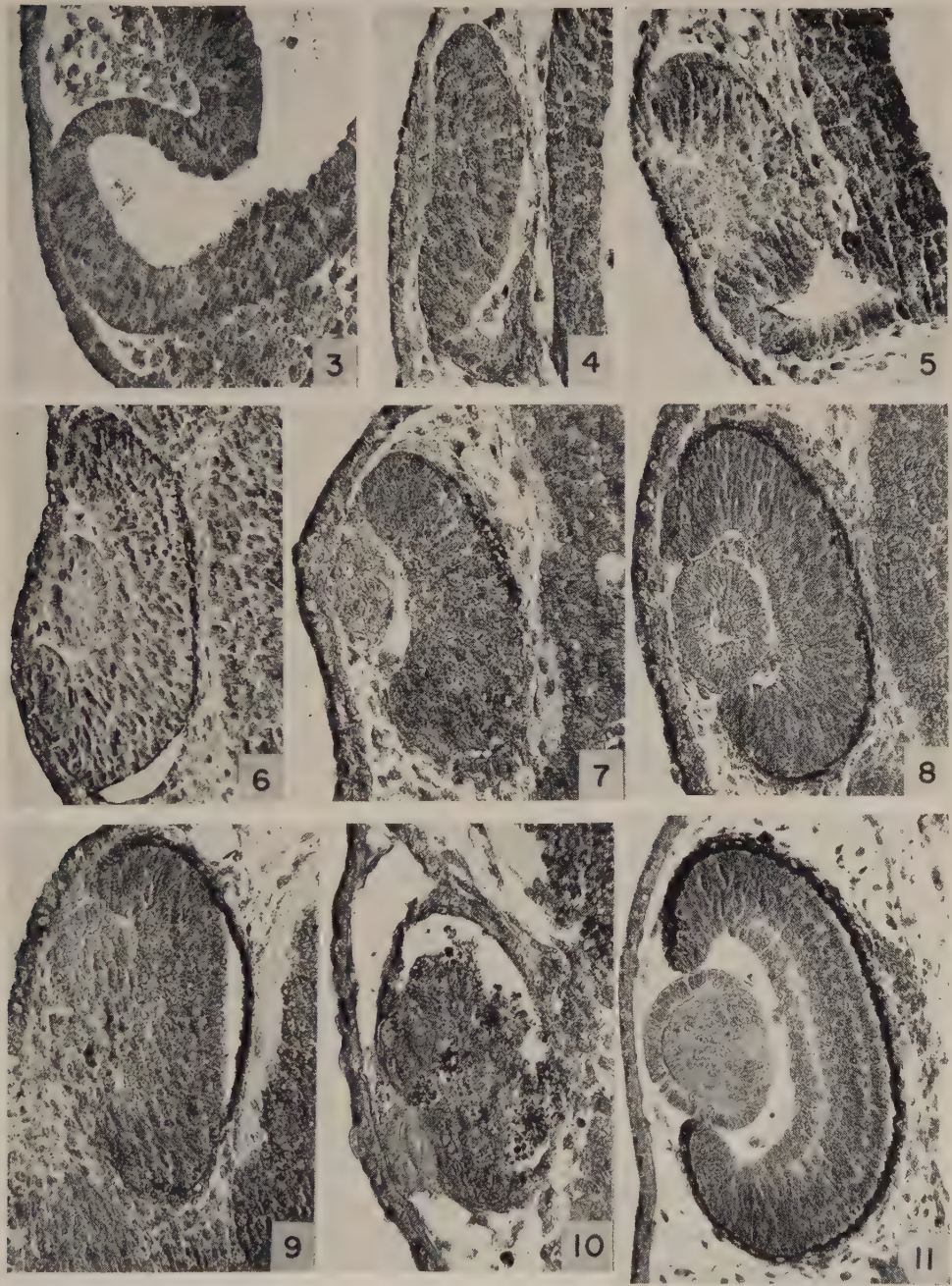


PLATE 2

EXPLANATION OF FIGURES

- 12 Normal primitive blood cells at stage 21, three days after start of experiment (cf. fig. 14). $\times 400$.
- 13 Cross section through the eye region of an embryo 6 days after exposure for 10 hours to 0.1 mg/ml of MNB. The embryo has recovered; note, however, the delay in development compared to control (fig. 15). $\times 50$.
- 14 Abnormal blood cells of an embryo 6 days after exposure for 20 hours to 0.1 mg/ml MNQ. Compare with normal blood cells of the same age (6 days, fig. 17) and younger normal blood cells (three days, fig. 12). It is seen that some cells and nuclei (a) are of the same size as those of younger controls; others have enlarged lobular nuclei (b); others have fragmented nuclei and the total cell volume is also larger (c). $\times 400$.
- 15 Cross section through the eye region of a normal control embryo at stage 25, 6 days after start of the experiment (cf. fig. 13). $\times 50$.
- 16 Cross section through the eye region of a severely arrested embryo 6 days after exposure for 20 hours to 0.1 mg/ml MNB. Note that the eye and lens are more arrested than the brain which has differentiated grey and white matter. Note also that there is no vascular tissue between the eye and the brain, because the circulatory system is arrested (cf. figs. 15 and 22). $\times 50$.
- 17 Normal blood cells of stage 25, 6 days after start of experiment (cf. figs. 12 and 14). $\times 400$.
- 18 Cross section through the normal ear region at stage 25, 6 days after start of experiment. $\times 50$.
- 19 Cross section of an embryo 6 days after exposure for 20 hours to 0.1 mg/ml MNQ. The ears are arrested as cystic sacs (cf. fig. 18). $\times 50$.

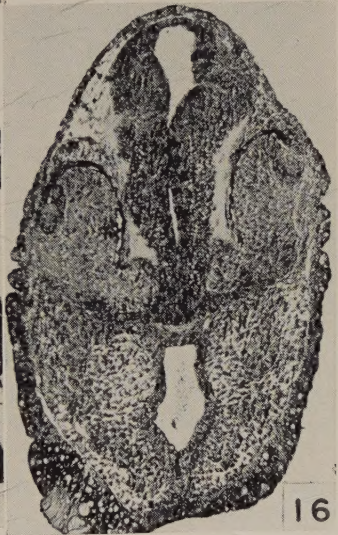
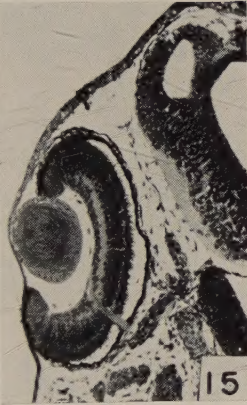
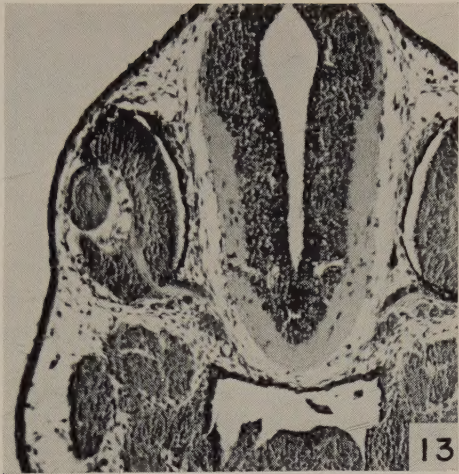
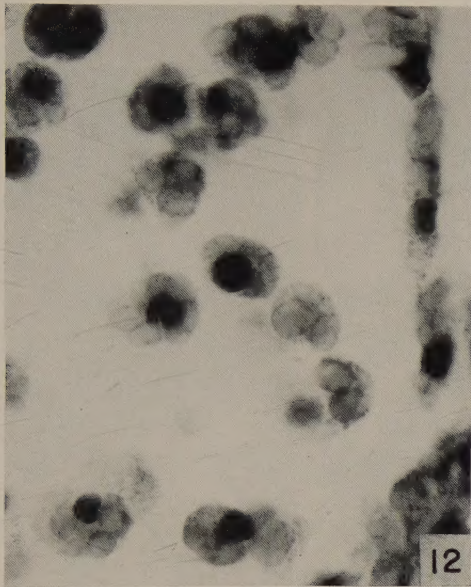


PLATE 3

EXPLANATION OF FIGURES

- 20 Cross section through the region of the heart of an embryo, three days after exposure for 20 hours to 0.1 mg/ml MNQ at 18–20°C. Note that the development of the heart and the primitive blood cells lag behind the controls (fig. 21) and have remained at a younger stage shown in figure 24. $\times 67$.
- 21 Cross section through the region of the heart of a control embryo, 18–20°C., three days after start of the experiment (cf. fig. 20). $\times 67$.
- 22 Cross section through the region of the heart of a severely arrested embryo 6 days after exposure for 20 hours to 0.1 mg/ml MNB (cf. fig. 16 and 6 day control fig. 27). The heart is without a lumen, a solid mass of cells. No cellular enlargement and necrosis is found in the medulla as after exposure to MNQ and shown in figure 20. $\times 67$.
- 23 Cross section through the arrested atrophied heart of an embryo 6 days after exposure for 20 hours to 0.1 mg/ml MNQ (cf. control fig. 27). $\times 67$.
- 24 Cross section through the region of the heart of a control embryo developing at 15–16°C., three days after start of the experiment. $\times 67$.
- 25 Cross section through the heart of an embryo 6 days after exposure for 10 hours to 0.1 mg/ml MNB. A primitive lumen and a few blood elements are present (cf. figs. 9 and 27). $\times 67$.
- 26 Cross section through the somite region of an embryo 6 days after exposure for 20 hours to 0.1 mg/ml MNQ. Note degenerating giant muscle cells. $\times 67$.
- 27 Cross section through the heart region of a control embryo at stage 25, 6 days after start of the experiment. $\times 67$.

